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(54) Title: RECOMBINANT NEGATIVE STRAND RNA VIRUS EXPRESSION SYSTEMS AND VACCINES

(57) Abstract

Recombinant negative strand virus RNA templates which may be used to express heterologous gene products and/or to construct chimeric viruses are described. Influenza viral polymerase, which was prepared depleted of viral RNA, was used to copy small RNA templates prepared from plasmid-encoded sequences. Template constructions containing only the 3′ end of genomic RNA were shown to be efficiently copied, indicative that the promoter lay solely within the 15 nucleotide 3′ terminus. Sequences not specific for the influenza viral termini were not copied, and, surprisingly, RNAs containing termini identical to those from plus sense cRNA were copied at low levels. The specificity for recognition of the virus-sense promoter was further defined by site-specific mutagenesis. It was also found that increased levels of viral protein were required in order to catalyze both the cap-endonuclease primed and primer-free RNA synthesis from these model templates as well as from genomic length RNAs. This indicated that this reconstituted system had catalytic properties very similar to those of native viral RNPs. High levels of expression of a heterologous gene was obtained using the constructs and methods described.

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RECOMBINANT NEGATIVE STRAND RNA VIRUS EXPRESSION SYSTEMS AND VACCINES

1. INTRODUCTION

The present invention relates to recombinant negative strand virus RNA templates which may be used to express heterologous gene products in appropriate host cell systems and/or to construct recombinant viruses that express, package, and/or present the heterologous gene product. The expression products and chimeric viruses may advantageously be used in vaccine formulations.

The invention is demonstrated by way of examples in which recombinant influenza virus RNA templates containing a heterologous gene coding sequences in the negative-polarity were constructed. These recombinant templates, when combined with purified viral RNA-directed RNA polymerase, were infectious, replicated in appropriate host cells, and expressed the heterologous gene product at high levels. In addition, the heterologous gene was expressed and packaged by the resulting recombinant influenza viruses.

2. BACKGROUND OF THE INVENTION

A number of DNA viruses have been genetically engineered to direct the expression of heterologous proteins in host cell systems (e.g., vaccinia virus, baculovirus, etc.). Recently, similar advances have been made with positive-strand RNA viruses (e.g., poliovirus). The expression products of these constructs, i.e., the heterologous gene product or the chimeric virus which expresses the heterologous gene product, are thought to be potentially useful in vaccine formulations (either subunit or whole virus vaccines). One drawback to the use of viruses such as vaccinia for constructing recombinant or chimeric viruses for use in vaccines is the lack of

variation in its major epitopes. This lack of variability in the viral strains places strict limitations on the repeated use of chimeric vaccinia, in that multiple vaccinations will generate host-resistance to the strain so that the inoculated virus cannot infect the host. Inoculation of a resistant individual with chimeric vaccina will, therefore, not induce immune stimulation.

By contrast, influenza virus, a negative-strand RNA virus, demonstrates a wide variability of its major epitopes. Indeed, thousands of variants of influenza have been identified; each strain evolving by antigenic drift. The negative-strand viruses such as influenza would be attractive candidates for constructing chimeric viruses for use in vaccines because its genetic variability allows for the construction of a vast repertoire of vaccine formulations which will stimulate immunity without risk of developing a tolerance. However, achieving this goal has been precluded by the fact that, to date, it has not been possible to construct recombinant or chimeric negative-strand RNA particles that are infectious.

2.1. THE INFLUENZA VIRUS

Virus families containing enveloped single-stranded RNA of the negative-sense genome are classified into groups having non-segmented genomes (Paramyxoviridae, Rhabdoviridae) or those having segmented genomes (Orthomyxoviridae, Bunyaviridae and Arenaviridae). The Orthomyxoviridae family, described in detail below, and used in the examples herein, contains only the viruses of influenza, types A, B and C.

The influenza virions consist of an internal ribonucleoprotein core (a helical nucleocapsid) containing the single-stranded RNA genome, and an outer lipoprotein envelope lined inside by a matrix protein (M). The segmented genome of influenza A consists of eight

molecules (seven for influenza C) of linear, negative polarity, single-stranded RNAs which encode ten polypeptides, including: the RNA-directed RNA polymerase proteins (PB2, PB1 and PA) and nucleoprotein (NP) which form the nucleocapsid; the matrix proteins (M1, M2); two surface glycoproteins which project from the lipoprotein envelope: hemagglutinin (HA) and neuraminidase (NA); and nonstructural proteins whose function is unknown (NS1 and NS2). Transcription and replication of the genome takes place in the nucleus and assembly occurs via budding on the plasma membrane. The viruses can reassort genes during mixed infections.

Influenza virus adsorbs via HA to sialyloligosaccharides in cell membrane glycoproteins and glycolipids. Following endocytosis of the virion, a conformational change in the HA molecule occurs within the cellular endosome which facilitates membrane fusion, thus triggering uncoating. The nucleocapsid migrates to the nucleus where viral mRNA is transcribed as the essential initial event in infection. Viral mRNA is transcribed by a unique mechanism in which viral endonuclease cleaves the capped 5'-terminus from cellular heterologous mRNAs which then serve as primers for transcription of viral RNA templates by the viral transcriptase. Transcripts terminate at sites 15 to 22 bases from the ends of their templates, where oligo(U) sequences act as signals for the template-independent addition of poly(A) tracts. Of the eight viral mRNA molecules so produced, six are monocistronic messages that are translated directly into the proteins representing HA, NA, NP and the viral polymerase proteins, PB2, PB1 and PA. The other two transcripts undergo splicing, each yielding two mRNAs which are translated in different reading frames to produce M1, M2, NS1 and NS2. In other words, the eight viral mRNAs code for ten proteins: eight structural and

two nonstructural. A summary of the genes of the influenza virus and their protein products is shown in Table I below.

TABLE I
Influenza Virus Genome RNA Segments and Coding Assignments[a]

Segment	Length[b] (Nucleotides)	Encoded Polypeptide[c]	Length[d] (Amino Acids)	Molecules Per Virion	Comments
1	2341	PB2	759	30-60	RNA transcriptase component; host cell RNA cap binding
2	2341	PB1	757	30-60	RNA transcriptase component; initiation of transcription; endonuclease activity?
3	2233	PA	716	30-60	RNA transcriptase component; elongation of mRNA chains?
4	1778	HA	566	500	Hemagglutinin; trimer; envelope glycoprotein; mediates attachment to cells
5	1565	NP	498	1000	Nucleoprotein; associated with RNA; structural component of RNA transcriptase

Influenza Virus Genome RNA Segments and Coding Assignments[a]

Segment	Length[b] (Nucleotides)	Encoded Polypeptide[c]	Length[d] (Amino Acids)	Molecules Per Virion	Comments
6	1413	NA	454	100	Neuraminidase; tetramer; envelope glycoprotein
7	1027	M_1	252	3000	Matrix protein; lines inside of envelope
		M_2	96		Structural protein in plasma membrane; spliced mRNA
		?	?9		Unidentified protein
8	890	NS_1	230		Nonstructural protein; function unknown
		NS_2	121		Nonstructural protein; function unknown; spliced mRNA

[a] Adapted from R.A. Lamb and P. W. Choppin (1983), Reproduced from the Annual Review of Biochemistry, Volume 52, 467-506.

[b] For A/PR/8/34 strain

[c] Determined by biochemical and genetic approaches

[d] Determined by nucleotide sequence analysis and protein sequencing

Following transcription, virus genome replication is the second essential event in infection by negative-strand RNA viruses. As with other negative-strand RNA viruses, virus genome replication in influenza is mediated by virus-specified proteins. It is hypothesized that most or all of the viral proteins that transcribe influenza virus mRNA segments also carry out their replication. All viral RNA segments have common 3′ and 5′ termini, presumably to enable the RNA-synthesizing apparatus to recognize each segment with equal efficiency. The mechanism that regulates the alternative uses (i.e., transcription or replication) of the same complement of proteins (PB2, PB1, PA and NP) has not been clearly identified but appears to involve the abundance of free forms of one or more of the nucleocapsid proteins, in particular, the NP. The nucleus appears to be the site of virus RNA replication, just as it is the site for transcription.

The first products of replicative RNA synthesis are complementary copies (i.e., plus-polarity) of all influenza virus genome RNA segments (cRNA). These plus-stranded copies (anti-genomes) differ from the plus-strand mRNA transcripts in the structure of their termini. Unlike the mRNA transcripts, the anti-genomic cRNAs are not capped and methylated at the 5′ termini, and are not truncated and polyadenylated at the 3′ termini. The cRNAs are coterminal with their negative strand templates and contain all the genetic information in each genomic RNA segment in the complementary form. The cRNAs serve as templates for the synthesis of genomic negative-strand vRNAs.

The influenza virus negative strand genomes (vRNAs) and antigenomes (cRNAs) are always encapsidated by nucleocapsid proteins; the only unencapsidated RNA species are virus mRNAs. In contrast to the other enveloped RNA viruses, nucleocapsid assembly appears to take place in the nucleus rather than in the cytoplasm. The virus matures by

budding from the apical surface of the cell incorporating the M protein on the cytoplasmic side or inner surface of the budding envelope. The HA and NA become glycosylated and incorporated into the lipid envelope. In permissive cells, HA is eventually cleaved, but the two resulting chains remain united by disulfide bonds.

It is not known by what mechanism one copy of each of the eight genomic viral RNAs is selected for incorporation into each new virion. Defective interfering (DI) particles are often produced, especially following infection at high multiplicity.

2.2. RNA DIRECTED RNA POLYMERASE

The RNA-directed RNA polymerases of animal viruses have been extensively studied with regard to many aspects of protein structure and reaction conditions. However, the elements of the template RNA which promote optimal expression by the polymerase could only be studied by inference using existing viral RNA sequences. This promoter analysis is of interest since it is unknown how a viral polymerase recognizes specific viral RNAs from among the many host-encoded RNAs found in an infected cell.

Animal viruses containing plus-sense genome RNA can be replicated when plasmid-derived RNA is introduced into cells by transfection (for example, Racaniello et al., 1981, Science 214:916-919; Levis, et al., 1986, Cell 44: 137-145). In the case of poliovirus, the purified polymerase will replicate a genome RNA in *in vitro* reactions and when this preparation is transfected into cells it is infectious (Kaplan, et al., 1985, Proc. Natl. Acad. Sci. USA 82:8424-8428). However, the template elements which serve as transcription promoter for the poliovirus-encoded polymerase are unknown since even RNA homopolymers can be copied (Ward, et al., 1988, J. Virol. 62: 558-562). SP6 transcripts have also been used to produce model defective interfering (DI)

RNAs for the Sindbis viral genome. When the RNA is introduced into infected cells, it is replicated and packaged. The RNA sequences which were responsible for both recognition by the Sindbis viral polymerase and packaging of the genome into virus particles were shown to be within 162 nucleotides (nt) of the 5′ terminus and 19 nt of the 3′ terminus of the genome (Levis, et al., 1986, Cell 44: 137-145). In the case of brome mosaic virus (BMV), a positive strand RNA plant virus, SP6 transcripts have been used to identify the promoter as a 134 nt tRNA-like 3′ terminus (Dreher, and Hall, 1988, J. Mol. Biol. 201: 31-40). Polymerase recognition and synthesis were shown to be dependent on both sequence and secondary structural features (Dreher, et al., 1984, Nature 311: 171-175).

The negative-sense RNA viruses have been refractory to study of the sequence requirements of the replicase. The purified polymerase of vesicular stomatitis virus is only active in transcription when virus-derived ribonucleoprotein complexes (RNPs) are included as template (De and Banerjee, 1985, Biochem. Biophys. Res. Commun. 126: 40-49; Emerson and Yu, 1975, J. Virol. 15: 1348-1356; Naito, and Ishihama, 1976, J. Biol. Chem. 251: 4307-4314). RNPs have been reconstituted from naked RNA of VSV DI particles using infected cell extracts as protein source. These RNPs were then replicated when added back to infected cells (Mirakhur, and Peluso, 1988, Proc. Natl. Acad. Sci. USA 85: 7511-7515). With regard to influenza viruses, it was recently reported that naked RNA purified from virus was used to reconstitute RNPs. The viral nucleocapsid and polymerase proteins were gel-purified and renatured on the viral RNA using thioredoxin (Szewczyk, et al., 1988, Proc. Natl. Acad. Sci. USA, 85: 7907-7911). However, these authors did not show that the activity of the preparation was specific for influenza viral RNA, nor did they analyze the signals which promote transcription.

During the course of influenza virus infection the polymerase catalyzes three distinct transcription activities. These include the synthesis of (a) subgenomic mRNA, which contains a 5′ cap and a 3′ poly-A tail; (b) a full length plus-strand or anti-genome (cRNA) copied from the genome RNA; and (c) genomic vRNA synthesized from the full length cRNA (reviewed in Ishihama and Nagata, 1988, CRC Crit. Rev. Biochem. 23: 27-76; and Krug, Transcription and replication of influenza viruses. In: Genetics of influenza viruses, Ed., Palese, P. and Kingsbury, D.W. New York, Springer-Verlag, 1983, p. 70-98). Viral proteins PB2, PB1 and PA are thought to catalyze all influenza virus-specific RNA synthesis when in the presence of excess nucleocapsid protein (NP; see above reviews). These polymerase functions have been studied using RNP cores derived from detergent-disrupted virus, and RNPs from the nuclear extracts of infected cells. Transcription from the RNPs derived from disrupted virus occurs when primed with either dinucleotide adenylyl-(3′-5′)-guanosine (ApG) or capped mRNAs. The plus sense mRNA products have terminated synthesis 17-20 nucleotides upstream of the 5′ terminus of the RNA template and have been processed by the addition of poly A tails. These products cannot serve as template for the viral-sense genome since they lack terminal sequences (Hay, et al., 1977, Virology 83: 337-355). RNPs derived from nuclear extracts of infected cells also synthesize polyadenylated mRNA in the presence of capped RNA primers. However, if ApG is used under these conditions, both RNAs, polyadenylated and full length cRNA, can be obtained (Beaton and Krug, 1986, Proc. Natl. Acad. Sci. USA 83: 6282-6286; Takeuchi, et al., 1987, J. Biochem. 101: 837-845). Recently it was shown that replicative synthesis of cRNA could occur in the absence of exogenous primer if the nuclear extract was harvested at certain times post infection. In these same preparations the synthesis of negative-sense vRNA from a cRNA template was also observed

(Shapiro and Krug, 1988, J. Virol. 62: 2285-2290). The synthesis of full length cRNA was shown to be dependent upon the presence of nucleocapsid protein (NP) which was free in solution (Beaton and Krug, 1986, Proc. Natl. Acad. Sci. USA 83: 6282-6286; Shapiro and Krug, 1988, J. Virol. 62: 2285-2290). These findings led to the suggestion that the regulatory control between mRNA and cRNA synthesis by the RNP complex is based on the requirement for there being an excess of soluble NP (Beaton and Krug, 1986, Proc. Natl. Acad. Sci. USA 83: 6282-6286).

Another line of investigation has focused on the preparation of polymerase-RNA complexes derived from RNPs from detergent-disrupted virus. When the RNP complex is centrifuged through a CsCl-glycerol gradient, the RNA can be found associated with the three polymerase (P) proteins at the bottom of the gradient. Near the top of the gradient, free NP protein can be found (Honda, et al., 1988, J. Biochem. 104: 1021-1026; Kato, et al., 1985, Virus Research 3, 115-127). The purified polymerase-RNA complex (bottom of gradient), is active in initiating ApG-primed synthesis of RNA, but fails to elongate to more than 12-19 nucleotides. When fractions from the top of the gradient containing the NP protein are added back to the polymerase-RNA complex, elongation can ensue (Honda, et al., 1987, J. Biochem. 102: 41-49). These data suggest that the NP protein is needed for elongation, but that initiation can occur in the absence of NP.

It has been shown that the genomic RNA of influenza viruses is in a circular conformation via base-pairing of the termini to form a panhandle of 15 to 16 nt (Honda, et al., 1988, J. Biochem. 104: 1021-1026; Hsu, et al., 1987, Proc. Natl. Acad. Sci. USA 84: 8140-8144). Since the viral polymerase was found bound to the panhandle, this led to the suggestion that a panhandle structure was required for recognition by the viral polymerase (Honda, et al., 1988, J.

Biochem. 104: 1021-1026.) Therefore, it was hypothesized in these two reports that the promoter for the viral RNA polymerase was the double stranded RNA in panhandle conformation.

3. SUMMARY OF THE INVENTION

Recombinant negative-strand viral RNA templates are described which may be used with purified RNA-directed RNA polymerase complex to express heterologous gene products in appropriate host cells and/or to rescue the heterologous gene in virus particles. The RNA templates are prepared by transcription of appropriate DNA sequences with a DNA-directed RNA polymerase. The resulting RNA templates are of the negative-polarity and contain appropriate terminal sequences which enable the viral RNA-synthesizing apparatus to recognize the template.

As demonstrated by the examples described herein, recombinant negative-sense influenza RNA templates may be mixed with purified viral polymerase proteins and nucleoprotein (i.e., the purified viral polymerase complex) to form infectious recombinant RNPs. These can be used to express heterologous gene products in host cells or to rescue the heterologous gene in virus particles by cotransfection of host cells with recombinant RNPs and virus. Alternatively, the recombinant RNA templates or recombinant RNPs may be used to transfect transformed cell lines that express the RNA dependent RNA-polymerase and allow for complementation. Additionally, a non-virus dependent replication system for influenza virus is also described. Vaccinia vectors expressing influenza virus polypeptides were used as the source of proteins which were able to replicate and transcribe synthetically derived RNPs. The minimum subset of influenza virus protein needed for specific replication and expression of the viral RNP was found to be the three polymerase proteins (PB2, PB1 and PA) and the nucleoprotein

(NP). This suggests that the nonstructural proteins, NS1 and NS2, are not absolutely required for the replication and expression of viral RNP.

The expression products and/or chimeric virions obtained may advantageously be utilized in vaccine formulations. The use of recombinant influenza for this purpose is especially attractive since influenza demonstrates tremendous strain variability allowing for the construction of a vast repertoire of vaccine formulations. The ability to select from thousands of influenza variants for constructing chimeric viruses obviates the problem of host resistance encountered when using other viruses such as vaccinia. In addition, since influenza stimulates a vigorous secretory and cytotoxic T cell response, the presentation of foreign epitopes in the influenza virus background may also provide for the induction of secretory immunity and cell-mediated immunity.

3.1. DEFINITIONS

As used herein, the following terms will have the meanings indicated:

cRNA = anti-genomic RNA
HA = hemagglutinin (envelope glycoprotein)
M = matrix protein (lines inside of envelope)
MDCK = Madin Darby canine kidney cells
MDBK = Madin Darby bovine kidney cells
moi = multiplicity of infection
NA = neuraminidase (envelope glycoprotein)
NP = nucleoprotein (associated with RNA and required for polymerase activity)
NS = nonstructural protein (function unknown)
nt = nucleotide
PA, PB1, PB2 = RNA-directed RNA polymerase components
RNP = ribonucleoprotein (RNA, PB2, PB1, PA and NP)
rRNP = recombinant RNP

vRNA = genomic virus RNA
viral polymerase complex = PA, PB1, PB2 and NP
WSN = influenza A/WSN/33 virus
WSN-HK virus: reassortment virus containing seven genes from WSN virus and the NA gene from influenza A/HK/8/68 virus

4. DESCRIPTION OF THE FIGURES

Figure 1. Purification of the polymerase preparation. RNP cores were purified from whole virus and then subjected to CsCl-glycerol gradient centrifugation. The polymerase was purified from fractions with 1.5 to 2.0 M CsCl. Samples were then analyzed by polyacrylamide gel electrophoresis on a 7-14% linear gradient gel in the presence of 0.1% sodium dodecylsulfate followed by staining with silver. Protein samples contained 1.4 μg whole virus (lane 1), 0.3 μg whole virus (lane 2), 5 μl of RNP cores (lane 3) and 25 μl RNA polymerase (lane 4). Known assignments of the proteins are indicated at the left.

Figure 2. Plasmid constructs used to prepare RNA templates. The plasmid design is depicted with the solid box representing pUC-19 sequences, the hatched box represents the truncated promoter specifically recognized by bacteriophage T7 RNA polymerase, the solid line represents the DNA which is transcribed from plasmids which have been digested with MboII. The white box represents sequences encoding the recognition sites for MboII, EcoRI and PstI, in that order. Sites of cleavage by restriction endonucleases are indicated. Beneath the diagram, the entire sequences of RNAs which result from synthesis by T7 RNA polymerase from MboII-digested plasmid are given. The V-wt RNA has the identical 5′ and 3′ termini as found in RNA segment 8 of influenza A viruses, separated by 16 "spacer" nucleotides. The RNA, M-wt, represents the exact opposite stand, or "message-sense", of V-wt. Restriction endonuclease sites for DraI, EcoRI,

PstI and SmaI are indicated. T7 transcripts of plasmids cleaved by these enzymes result in, respectively, 32, 58, 66 and 91 nucleotide long RNAs. The sequences of V-d5′ RNA are indicated. The plasmid design is essentially the same as that used for the V-wt RNA except for the minor changes in the "spacer" sequence. The point mutants of V-d5′ RNAs which were studied are indicated in Table I.

Figure 3. Analysis of products of influenza viral polymerase. FIG. 3A: Polymerase reaction mixtures containing 0.4 mM ApG (lane 2) or no primer (lane 3) were electrophoresed on 8% polyacrylamide gels containing 7.7 M urea. FIG. 3B: The nascent RNA is resistant to single-stranded specific nuclease S1. Following the standard polymerase reaction, the solutions were diluted in nuclease S1 buffer (lane 1) and enzyme was added (lane 2). As control for S1 digestion conditions, radioactively labeled single-stranded V-wt RNA was treated with nuclease S1 (lane 3) or with buffer alone (lane 4). FIG. 3C: Ribonuclease T1 analayis of gel-purified reaction products. The reaction products of the viral polymerase using the V-wt RNA template was subjected to electrophoresis on an 8% polyacrylamide gel. The 53 nt band and the smaller transcript were excised and eluted from the gel matrix. These RNAs were digested with RNAse T1 and analyzed by electrophoresis on a 20% polyacrylamide gel containing 7.7 M urea. For comparison, T7 transcripts of M-wt and V-wt RNAs which had been synthesized in the presence of a α-^{32}P-UTP were also analyzed with RNAse T1. The predicted radiolabeled oliognucleotides of the control RNAs are indicated. Lane 1, 53 nucleotide full length (FL) product; lane 2, 40-45 nucleotide smaller (Sm) RNA product; lane 3, M-wt RNA labeled by incorporation of ^{32}P-UMP; and lane 4, V-wt RNA labeled as in lane 3.

Figure 4. Optimal reaction conditions for the viral polymerase. FIG. 4A: Reactions with V-wt template were assembled on ice and then incubated at the indicated

temperatures for 90 minutes. FIG. 4B: Reactions with the V-wt template were prepared in parallel with the indicated NaCl or KCl concentrations and were incubated at 30°C for 90 minutes. FIG. 4C: A single reaction with the V-wt template was incubated at 30°C, and at the indicated times, samples were removed and immediately processed by phenol-chloroform extraction. All gels contained 8% polyacrylamide with 7.7 M urea.

Figure 5. Template specificity of the viral polymerase. FIG. 5A: The viral polymerase reaction requires 3′ terminal promoter sequences. Different template RNAs were used in reactions under standard conditions. Lane 1, the V-Pst RNA, which is identical to V-wt except it has a 13 nt extension at the 3′ end; lane 2, V-Sma RNA, which has a 38 nt extension at the 3′ end; lane 3, V-wt RNA; lane 4, a DNA polynucleotide with identical sequence as the V-wt RNA; lane 5, an 80 nt RNA generated by bacteriophage T3 RNA polymerase transcription of a pIBI-31 plasmid digested with HindIII. The autoradiograph was overexposed in order to emphasize the absence of specific reaction products when these other templates were used. FIG. 5B: 10 ng of each template RNA were incubated with the viral polymerase and the products were then subjected to electrophoresis on 8% polyacrylamide gels containing 7.7 M urea. Lane 1, V-wt RNA; lane 2, V-Dra RNA; lane 3, V-Eco RNA; lane 4, M-wt RNA are shown; and lane 5, a 53nt marker oligonucleotide. For the exact sequence differences refer to FIG. 2 and Section 6.1 et seq.

Figure 6. The RNA promoter does not require a terminal panhandle. Polymerase reaction using two template RNAs. Each reaction contained 5 ng of V-wt RNA. As a second template the reactions contained 0 ng (lane 1), 0.6 ng (lane 2), and 3.0 ng (lane 3) of V-d5′ RNA. The resulting molar ratios are as indicated in the figure. The reaction products were analyzed on an 8% polyacrylamide gel in the presence of 7.7 M urea. Following densitometry analysis of

autoradiographs, the relative intensity of each peak was corrected for the amount of radioactive UMP which is incorporated in each product.

Figure 7. Specificity of promoter sequences. RNAs which lacked the 5′ terminus and contained point mutations (Table II) were compared with V-d5′ RNA in standard polymerase reactions. The right panel is from a separate reaction set. Quantitative comparisons is outlined in Table II.

Figure 8. High concentration polymerase preparations are active in cap-endonuclease primed and in primerless RNA synthesis reactions. FIG. 8A: Primer specificty of the high concentration enzyme. Radioactively synthesized 30 nt template is in lane 1. Reactions using 20 ng of V-d5′ RNA and 5 μl of viral polymerase contained as primer: no primer (lane 2); 100 ng BMV RNA (De and Banerjee, 1985, Biochem. Biophys. Res. Commun. 6:40-49) containing a cap 0 structure (lane 3); 100 ng rabbit globin mRNA, containing a cap 1 structure, (lane 4); and 0.4 mM ApG (lane 5). A lighter exposure of lane 5 is shown as lane 6. FIG. 8B: Nuclease S1 analysis of gel-purified RNAs. Products from reactions using as primer ApG (lanes 1 and 2); no primer (lanes 3 and 4); or globin mRNA (lanes 5 and 6) were electrophoresed in the absence of urea and the appropriate gel piece was excised and the RNA was eluted. This RNA was then digested with nuclease S1 (lanes 2, 4, and 6) and the products were denatured and analyzed on an 8% polyacrylamide gel containing 7.7 M urea.

Figure 9. Genomic length RNA synthesis from reconstitituted RNPs. Reaction products using 10 μl of polymerase and as template 890 nt RNA identical to the sequence of segment 8 of virus A/WSN/33 and RNA extracted from A/PR/8/34 virus were analyzed on a 4% polyacrylamide gel containing 7.7 M urea. In lane 1, the 890 nt template synthesized radioactively by T7 RNA polymerase is shown. The 890 nt plasmid-derived RNA was used as template in lanes 2,

3, 8 and 9. RNA extracted from virus was used as template in lanes 4, 5, 10 and 11. No template was used in lanes 6 and 7. No primer was used in lanes 2 to 5, and ApG was used as primer in lanes 6 to 11. Reaction products were treated with nuclease S1 in lanes 3, 5, 7, 9 and 11.

Figure 10. Diagrammatic representation of a PCR-directed mutagenesis method which can be used to replace viral coding sequences within viral gene segments.

Figure 11.(A). Diagrammatic representation of relevant portions of pIVCAT1. The various domains are labeled and are, from left to right; a truncated T7 promoter; the 5′ nontranslated end of influenza A/PR/8/34 virus segment 8 (22 nucleotides); 8 nucleotides of linker sequence; the entire CAT gene coding region (660 nucleotides) the entire 3′ nontranslated end of influenza A/PR/8/34 virus segment 8 (26 nucleotides); and linker sequence containing the HgaI restriction enzyme site. Relevant restriction enzyme sites and start and stop sites for the CAT gene are indicated. (B) The 716 base RNA product obtained following HgaI digestion and transcription of pIVACAT1 by T7 RNA polymerase. Influenza viral sequences are indicated by bold letters, CAT gene sequences by plain letters, and linker sequences by italics. The triplets -- in antisense orientation -- representing the initiation and termination codons of the CAT gene are indicated by arrow and underline, respectively.

Figure 12. RNA products of T7 polymerase transcription and in vitro influenza virus polymerase transcription. Lanes 1-4: polyacrylamide gel analysis of radiolabeled T7 polymerase transcripts from pIVACAT1, and pHgaNS. Lanes 5 and 6: Polyacrylamide gel analysis of the radiolabeled products of in vitro transcription by purified influenza A polymerase protein using unlabeled 1VACAT1 RNA and HgaNS RNA templates. Lane 1: HgaNS RNA of 80 nt. Lanes

2-4: different preparations of IVACAT1 RNA. Lane 5: viral polymerase transcript of IVACAT1 RNA. Lane 6: viral polymerase transcript of HgaNS RNA.

Figure 13. Schematic of the RNP-transfection and passaging experiments.

Figure 14. CAT assays of cells RNP-transfected with IVACAT1 RNA. (A) Time course of RNP-transfection in 293 cells. Cells were transfected at -1 hour with the recombinant RNP and infected with virus at 0 hour. Cells were harvested at the indicated time points and assayed for CAT activity. (B) Requirements for RNP-transfection of 293 cells Paramaeters of the reaction mixtures were as indicated. (C) RNP-transfection of MDCK cells. MDCK cells were transfected with IVACAT1 RNA-polymerase at either -1 hour or +2 hours relative to virus infection. Cells were harvested and CAT activity assayed at the indicated times. Components/conditions of the reaction were as indicated. "Time" indicates the time point of harvesting the cells. T= 0 marks the time of addition of helper virus. "RNA" represents the IVACAT1 RNA. "Pol" is the purified influenza A/PR/8/34 polymerase protein complex. "WSN" indicates the influenza A/WSN/33 helper virus. "Pre-Inc." indicates preincubation of RNA and polymerase in transcription buffer at 30° C for 30 min. "RNP transfection" indicates the time of RNP transfection relative to virus infection. "+/-" indicate presence or absence of the particular component/feature. "C" indicates control assays using commercially available CAT enzyme (Boehringer-Mannheim).

Figure 15. CAT activity in MDCK cells infected with recombinant virus. Supernatant from RNP-transfected and helper virus-infected MDCK cells was used to infect fresh MDCK cells. The inoculum was removed 1 hour after infection, cells were harvested 11 hours later and CAT activty was

assayed. Lane 1: extract of cells infected with helper virus only. Lane 2: extract of cells infected with 100 μl of supernatant from RNP-transfected and helper virus-infected MDCK cells. Lane 3: Supernatant (80 μl) of cells from lane 2. Lane 4: Same as lane 2 except that helper virus (MOI 4) was added to inoculum. In contrast to experiments shown in FIG. 4, the assays contained 20 μl of ^{14}C chloramphenicol.

FIG. 16 Diagram of relevant portions of the neuraminidase (NA) gene contained in plasmids used for transfection experiments. The pUC19 derived plasmid pT3NAv contains the influenza A/WSN/33 virus NA gene and a truncated promoter specifically recognized by bacteriophage T3 RNA polymerase. The T3 promoter used is truncated such that the initial transcribed nucleotide (an adenine) corresponds to the 5′ adenine of the WSN NA gene. At the 3′ end of the cDNA copy of the NA gene, a Ksp632I restriction enzyme site was inserted such that the cleavage site occurs directly after the 3′ end of the NA gene sequence. A 1409 nucleotide long transcript was obtained following Ksp632I digestion and transcription by T3 RNA polymerase of PT3NAv (as described in Section 8.1, infra). The 15 5′ terminal nucleotides, the 52 nucleotides corresponding to the region between the restriction endonuclease sites NcoI and PstI and the 12 3′ terminal nucleotides are shown. The transcript of pT3NAv mut 1 is identical to that of pT3NAv except for a single deletion, eleven nucleotides downstream from the 5′ end of the wild type RNA. The transcript of the pT3NAv mut 2 is identical to that of pT3NAv except for 5 mutations located in the central region (indicated by underline). These five mutations do not change the amino acid sequence in the open reading frame of the gene. The serine codon UCC at position 887-889 (plus sense RNA) was replaced with the serine codon AGU in the same frame. The numbering of nucleotides follows Hiti et al., 1982, J. Virol. 41:730-734.

FIG. 17. Polyacrylamide gel electrophoresis of RNAs purified from rescued influenza viruses. RNA transcripts of pT3NAs (FIG. 16) of phenol-extracted RNA derived from influenza A/WSN/33 virus was mixed with purified polymerase preparations following the protocol described in Section 6.1.1, infra. These reconstituted RNPs were then transfected into MDBK cells which had been infected one hour earlier with WSN-HK helper virus. The medium, containing 28 μg/ml plasminogen, was harvested after 16 hours and virus was amplified and plaqued on MDBK cells in the absence of protease. Virus obtained from plaques was then further amplified in MDBK cells and RNA was phenol-extracted from purified virus preparations as described in Sections 6.1 et seq. and 7.1 et seq. RNAs were separated on 2.8% polyacrylamide-0.075% bisacrylamide gels containing 7.7 M urea in TBE buffer and visualized by silverstaining as described in Section 6.1 et seq. Lanes 1 and 6: WSN-HK virus RNA. Lane 2: RNA of virus which was rescued from MDBK cells following RNP-transfection with pT3NAv derived NA RNA and infection with helper virus WSN-HK. Lane 3: NA RNA transcribed in vitro from pT3NAv. Lane 4: RNA of control WSN virus. Lane 5: RNA of virus which was rescued from MDBK cells following RNP-transfection with phenol-extracted WSN virus RNA and infection with helper virus WSN-HK.

FIG. 18. Sequence analysis of RNA obtained from rescued influenza virus containing five site-specific mutations. Following infection with the WSN-HK helper virus, MDBK cells were RNP-transfected with T3NAv mut 2 RNA which was obtained by transcription from pT3NAv mut 2. Following overnight incubation in the presence of 28 μg/ml plasminogen, medium was used for propagation and plaquing on MDBK cells in the absence of protease. Virus from plaques was then amplified and RNA was obtained following phenol-extraction of purified virus. Rescue of the mutant NA gene into virus particles was verified through direct RNA sequencing using

5'-TACGAGGAAATGTTCCTGTTA-3' as primer (corresponding to position 800-819; Hiti et al., J. Virol. 41:730-734) and reverse transcriptase (Yamashita et al., 1988, Virol. 163:112-122). Sequences shown correspond to position 878-930 in the NA gene (Hiti et al., J. Virol. 41:730-734). The arrows and the underlined nucleotides indicate the changes in the mutant RNA compared to the wild type RNA. Left: Control RNA obtained from influenza A/WSN/33 virus. Right: RNA of mutant virus rescued from MDBK cells which were RNP-transfected with T3NAv mut 2 RNA and infected with helper virus WSN-HK.

FIG. 19. CAT expression in vaccinia virus-infected/IVACAT-1 RNP transfected cells. Approximately 10^6 mouse C127 cells in 35 mm dishes were infected with mixtures of recombinant vaccinia viruses (Smith et al., 1986) at an M.O.I. of approximately 10 for each vector. After 1.5 hours, synthetic IVACAT-1 RNP was transfected into the virus-infected cells as described (Lutjyes et al., 1989). Cells were incubated overnight, harvested and assayed for CAT activity according to standard procedures (Gorman et al., 1982). The assays contained .05 uCl [^{14}C] chloramphenicol, 20 ul of 40 mM acetyl-CoA (Boehringer and 50 ul of cell extracts in 0.25 M Tris buffer (pH 7.5). Incubation times were approximately 4 hours. The labels under the lane numbers indicate the treatment of cells. Lanes 1-control; 2-naked RNA transfection (no polymerase added), no helper virus infection; 3-RNP transfection, no helper virus; 4-RNP transfection, influenza virus as helper; Lanes 5-11-RNP transfection, vaccinia virus vectors as helper viruses express the indicated influenza virus proteins.

FIG. 20. Test of various cell lines. A) Cells were infected with vaccinia vectors expressing the PB2, PB1 and PA proteins (Lanes 1,3,5,7) or the PB2, PB1, PA and NP proteins (Lanes 2,4,6,8), transfected with IVACAT-1 RNP and examined for CAT activity as described. Lanes 1,2: Maden-Darby Canine

Kidney (MDCK) cell; 3,4: Hela cells, 5,6: 293 cells (Graham et al., 1977 J. gen. Virol 36: 59-72); 7,8 L cells. B) Cell line 3 PNP-4 was used as host cell. Shown under each lane is the influenza viral proteins expressed in each sample. C) 293 cells were infected with the four required vaccinia and transfected with synthetic RNP made using IVA-CAT-1 (lane 1) or IVA-CAT-2 (lane 2) RNA. After overnight incubation, cells were harvested and CAT assayss were performed.

5. DESCRIPTION OF THE INVENTION

This invention relates to the construction and use of recombinant negative strand viral RNA templates which may be used with viral RNA-directed RNA polymerase to express heterologous gene products in appropriate host cells and/or to rescue the heterologous gene in virus particles. The RNA templates may be prepared by transcription of appropriate DNA sequences using a DNA-directed RNA polymerase such as bacteriophage T7, T3 or the Sp6 polymerase. Using influenza, for example, the DNA is constructed to encode the message-sense of the heterologous gene sequence flanked upstream of the ATG by the complement of the viral polymerase binding site/promoter of influenza, i.e., the complement of the 3'-terminus of a genome segment of influenza. For rescue in virus particles, it may be preferred to flank the heterologous coding sequence with the complement of both the 3'-terminus and the 5'-terminus of a genome segment of influenza. After transcription with a DNA-directed RNA polymerase, the resulting RNA template will encode the negative polarity of the heterologous gene sequence and will contain the vRNA terminal sequences that enable the viral RNA-directed RNA polymerase to recognize the template.

The recombinant negative sense RNA templates may be mixed with purified viral polymerase complex comprising viral RNA-directed RNA polymerase proteins (the P proteins) and nucleoprotein (NP) which may be isolated from RNP cores

prepared from whole virus to form "recombinant RNPs" (rRNPs). These rRNPs are infectious and may be used to express the heterologous gene product in appropriate host cells or to rescue the heterologous gene in virus particles by cotransfection of host cells with the rRNPs and virus. Alternatively, the recombinant RNA templates may be used to transfect transformed cell lines that express the RNA-directed RNA polymerase proteins allowing for complementation.

The invention is demonstrated by way of working examples in which RNA transcripts of cloned DNA containing the coding region -- in negative sense orientation -- of the chloramphenicol acetyltransferase (CAT) gene, flanked by the the 22 5′ terminal and the 26 3′ terminal nucleotides of the influenza A/PR/8/34 virus NS RNA were mixed with isolated influenza A virus polymerase proteins. This reconstituted ribonucleoprotein (RNP) complex was transfected into MDCK (or 293) cells, which were infected with influenza virus. CAT activity was negligible before and soon after virus infection, but was demonstrable by seven hours post virus infection. When cell supernatant containing budded virus from this "rescue" experiment was used to infect a new monolayer of MDCK cells, CAT activity was also detected, suggesting that the RNA containing the recombinant CAT gene had been packaged into virus particles. These results demonstrate the successful use of recombinant negative strand viral RNA templates and purified RNA-dependent RNA polymerase to reconstitute recombinant influenza virus RNP. Furthermore, the data suggest that the 22 5′ terminal and the 26 3′ terminal sequences of the influenza A virus RNA are sufficient to provide the signals for RNA tanscription, RNA replication and for packaging of RNA into influenza virus particles.

Using this methodology we also demonstrated the rescue of synthetic RNAs, derived from appropriate recombinant plasmid DNAs, into stable and infectious influenza viruses. In particular, RNA corresponding to the neuraminidase (NA) gene of influenza A/WSN/33 virus (WSN) was transcribed in vitro from plasmid DNA and, following the addition of purified influenza virus polymerase complex, was transfected into MDBK cells. Superinfection with helper virus lacking the WSN NA gene resulted in the release of virus containing the WSN NA gene. We then introduced five point mutations into the WSN NA gene by cassette mutagenesis of the plasmid DNA. Sequence analysis of the rescued virus revealed that the genome contained all five mutations present in the mutated plasmid. This technology can be used to create viruses with site-specific mutations so that influenza viruses with defined biological properties may be engineered.

The ability to reconstitute RNP's in vitro allows the design of novel chimeric influenza viruses which express foreign genes. One way to achieve this goal involves modifying existing influenza virus genes. For example, the HA gene may be modified to contain foreign sequences in its external domains. Where the heterologous sequence are epitopes or antigens of pathogens, these chimeric viruses may be used to induce a protective immune response against the disease agent from which these determinants are derived. In addition to modifying genes coding for surface proteins, genes coding for nonsurface proteins may be altered. The latter genes have been shown to be associated with most of the important cellular immune responses in the influenza virus system (Townsend et al., 1985, Cell 42:475-482). Thus, the inclusion of a foreign determinant in the NP or the NS gene of an influenza virus may - following infection - induce an effective cellular immune response against this determinant. Such an approach may be particularly helpful in

situations in which protective immunity heavily depends on the induction of cellular immune responses (e.g., malaria, etc.).

Another approach which would permit the expression of foreign proteins (or domains of such proteins) via chimeric influenza viruses concerns the introduction of complete heterologous genes into the virus. Influenza virus preparations with more than eight RNA segments have previously been described (Nayak, D. et al. in Genetics of Influenza Virus, P. Palese and D. W. Kingsbury, eds., Springer-Verlag, Vienna, pp. 255-279). Thus, chimeric influenza viruses with nine or more RNA segments may be viable, and correct packaging of such chimeric viruses may readily occur.

The invention may be divided into the following stages solely for the purpose of description and not by way of limitation: (a) construction of recombinant RNA templates; (b) expression of heterologous gene products using the recombinant RNA templates; and (c) rescue of the heterologous gene in recombinant virus particles. For clarity of discussion, the invention is described in the subsections below using influenza. Any strain of influenza (e.g., A, B, C) may be utilized. However, the principles may be analagously applied to construct other negative strand RNA virus templates and chimeric viruses including, but not limited to paramyxoviruses, such as parainfluenza viruses, measles viruses, respiratory syncytial virus; bunyaviruses; arena viruses; etc. A particularly interesting virus system that can be used in accordance with the invention are the orthomyxo-like insect virus called Dhori (Fuller, 1987, Virology 160:81-87).

5.1. CONSTRUCTION OF THE RECOMBINANT RNA TEMPLATES

Heterologous gene coding sequences flanked by the complement of the viral polymerase binding site/promoter, e.g, the complement of 3'-influenza virus terminus, or the complements of both the 3'- and 5'-influenza virus termini may be constructed using techniques known in the art. Recombinant DNA molecules containing these hybrid sequences can be cloned and transcribed by a DNA-directed RNA polymerase, such as bacteriophage T7, T3 or the Sp6 polymerase and the like, to produce the recombinant RNA templates which possess the appropriate viral sequences that allow for viral polymerase recognition and activity.

One approach for constructing these hybrid molecules is to insert the heterologous coding sequence into a DNA complement of an influenza virus genomic segment so that the heterologous sequence is flanked by the viral sequences required for viral polymerase activity; i.e., the viral polymerase binding site/promoter, hereinafter referred to as the viral polymerase binding site. In an alternative approach, oligonucleotides encoding the viral polymerase binding site, e.g., the complement of the 3'-terminus or both termini of the virus genomic segments can be ligated to the heterologous coding sequence to construct the hybrid molecule. The placement of a foreign gene or segment of a foreign gene within a target sequence was formerly dictated by the presence of appropriate restriction enzyme sites within the target sequence. However, recent advances in molecular biology have lessened this problem greatly. Restriction enzyme sites can readily be placed anywhere within a target sequence through the use of site-directed mutagenesis (e.g., see, for example, the techniques described by Kunkel, 1985, Proc. Natl. Acad. Sci. U.S.A. 82;488). Variations in polymerase chain reaction (PCR) technology, described infra, also allow for the specific insertion of sequences (i.e., restriction enzyme sites) and allow for the

facile construction of hybrid molecules. Alternatively, PCR reactions could be used to prepare recombinant templates without the need of cloning. For example, PCR reactions could be used to prepare double-stranded DNA molecules containing a DNA-directed RNA polymerase promoter (e.g., bacteriophase T3, T7 or Sp6) and the hybrid sequence containing the heterologous gene and the influenza viral polymerase binding site. RNA templates could then be transcribed directly from this recombinant DNA. In yet another embodiment, the recombinant RNA templates may be prepared by ligating RNAs specifying the negative polarity of the heterologous gene and the viral polymerase binding site using an RNA ligase. Sequence requirements for viral polymerase activity and constructs which may be used in accordance with the invention are described in the subsections below.

5.1.1. THE VIRAL 3'-TERMINUS IS REQUIRED FOR POLYMERASE ACTIVITY

The experiments described in Section 6 et seq., infra, are the first to define promoter sequences for a polymerase of a negative-sense RNA virus, and it was found that the specificity lies in the 3' terminal 15 nucleotides. These viral polymerase binding site sequences, as well as functionally equivalent sequences may be used in accordance with the invention. For example, functionally equivalent sequences containing substitions, insertions, deletions, additions or inversions which exhibit similar activity may be utilized. The RNA synthesis by the viral polymerase described infra is a model for specific recognition and elongation by the influenza viral polymerase for the following reasons: (a) the polymerase has high activity when primed with ApG, a feature unique to influenza viral polymerase; (b) it has optimal activity at temperature and ionic conditions previously shown to be effective for the

viral RNPs; (c) the polymerase is specific for influenza viral sequences on the model RNA templates; (d) the polymerase is active in the cap-endonuclease primed RNA synthesis which is the hallmark of the influenza viral polymerase; (e) recognition of cap donor RNA is specific to cap 1 structures; and (f) genomic RNA segments are specifically copied.

5.1.2. A TERMINAL PANHANDLE IS NOT REQUIRED FOR OPTIMAL RECOGNITION AND SYNTHESIS BY THE VIRAL POLYMERASE

We had previously shown that the influenza viral segment RNAs base-pair at their termini to form panhandle structures. This was achieved by two methods. A cross-linking reagent derivative of psoralen covalently bound the termini of each segment in intact virus or in RNPs from infected cells (Hsu et al., 1987, Proc. Natl. Acad. Sci. USA 84: 8140-8144). The treated RNA was seen by electron microscopy to be circular, by virtue of the crosslinked termini. Similarly, the RNA termini in RNPs were found to be sensitive to ribonuclease V1, which recognizes and cleaves double-stranded RNA, and the viral polymerase was found to be bound to both termini in the panhandle conformation (Honda, et al., 1988, J. Biochem. 104: 1021-1026). In these studies the panhandle structure of the genomic RNA was shown to exist, and it was inferred to play a role in polymerase recognition. Although the template RNAs used in the examples described, were originally prepared to reveal panhandle-specific protein binding, it was found that the terminal panhandle had no obvious role in the polymerase reactions studied herein.

5.1.3. THE RNA POLYMERASE PREPARATION SPECIFICALLY COPIES NEGATIVE SENSE TEMPLATES

The viral polymerase was shown to synthesize RNA with optimal efficiency if the template had the "wild-type" negative sense 3′ terminus. It was shown that RNAs of unrelated sequence were not copied, and that those with extra polylinker sequences on the 3′ end were much less efficiently copied. A DNA of the correct sequence was similarly unsuitable as a template. The reaction was highly specific since the M-wt template was replicated only at very low levels. Even though our source of polymerase was intact virus, this finding was very surprising since it had never been suggested that the polymerase which recognizes the viral sense RNA would not efficiently copy the plus sense strand. Studies are underway to examine the specificity of the polymerase purified from infected cells at times post infection when the complementary RNA is copied into genomic templates. The present data support a model whereby the viral polymerase which copies vRNA is functionally different from that which synthesizes vRNA from cRNA by virtue of their promoter recognition. It is possible that by regulated modification of the polymerase in infected cells it then becomes capable of recognizing the 3′ terminus of plus sense RNA. By analyzing promoter mutants we investigated the fine specificity of the reaction and found that the only single mutation which generated a significantly lower level of synthesis was that of V-A_3 RNA. Furthermore, combinations of two or more point changes in positions 3, 5, 8 and 10 greatly lowered synthesis levels.

5.1.4. INSERTION OF THE HETEROLOGOUS GENE SEQUENCE INTO THE PB2, PB1, PA OR NP GENE SEGMENTS

The gene segments coding for the PB2, PB1, PA and NP proteins contain a single open reading frame with 24-45 untranslated nucleotides at their 5′-end, and 22-57 untranslated nucleotides at their 3′-end. Insertion of a foreign gene sequence into any of these segments could be

accomplished by either a complete replacement of the viral coding region with the foreign gene or by a partial replacement. Complete replacement would probably best be accomplished through the use of PCR-directed mutagenesis. The principle of this mutagenesis method is illustrated in FIG. 10. Briefly, PCR-primer A would contain, from 5′ to 3′, a unique restriction enzyme site, such as a class IIS restriction enzyme site (i.e., a "shifter" enzyme; that recognizes a specific sequence but cleaves the DNA either upstream or downstream of that sequence); the entire 3′ untranslated region of the influenza gene segment; and a stretch of nucleotides complementary to the carboxy-terminus coding portion of the foreign gene product. PCR-primer B would contain from the 5′ to 3′ end: a unique restriction enzyme site; a truncated but active phage polymerase sequence; the complement of the entire 5′ untranslated region of the influenza gene segment (with respect to the negative sense vRNA); and a stretch of nucleotides corresponding to the 5′ coding portion of the foreign gene. After a PCR reaction using these primers with a cloned copy of the foreign gene, the product may be excised and cloned using the unique restriction sites. Digestion with the class IIS enzyme and transcription with the purified phage polymerase would generate an RNA molecule containing the exact untranslated ends of the influenza viral gene segment with a foreign gene insertion. Such a construction is described for the chloramphenicol acetyltransferase (CAT) gene used in the examples described in Section 7 infra. In an alernate embodiment, PCR-primed reactions could be used to prepare double-stranded DNA containing the bacteriophage promoter sequence, and the hybrid gene sequence so that RNA templates can be transcribed directly without cloning.

Depending on the integrity of the foreign gene product and the purpose of the construction, it may be desirable to construct hybrid sequences that will direct the expression of

fusion proteins. For example, the four influenza virus proteins, PB2, PB1, PA or NP are polymerase proteins which are directed to the nucleus of the infected cell through specific sequences present in the protein. For the NP this amino acid sequence has been found to be (single letter code) QLVWMACNSAAFEDLRVLS (Davey et al., 1985, Cell 40:667-675). Therefore, if it is desired to direct the foreign gene product to the nucleus (if by itself it would not ordinarily do so) the hybrid protein should be engineered to contain a domain which directs it there. This domain could be of influenza viral origin, but not necessarily so. Hybrid proteins can also be made from non-viral sources, as long as they contain the necessary sequences for replication by influenza virus (3' untranslated region, etc.).

As another example, certain antigenic regions of the viral gene products may be substituted with foreign sequences. Townsend et al., (1985, Cell 42:475-482), identified an epitope within the NP molecule which is able to elicit a vigorous CTL (cytotoxic T cell) response. This epitope spans residues 147-161 of the NP protein and consists of the amino acids TYQRTRQLVRLTGMDP. Substituting a short foreign epitope in place of this NP squence may elicit a strong cellular immune response against the intact foreign antigen. Conversely, expression of a foreign gene product containing this 15 amino acid region may also help induce a strong cellular immune response against the foreign protein.

5.1.5. INSERTION OF THE HETEROLOGOUS GENE SEQUENCE INTO THE HA OR NA GENE SEGMENTS

The HA and NA proteins, coded for by separate gene segments, are the major surface glycoproteins of the virus. Consequently, these proteins are the major targets for the humoral immune response after infection. They have been the

most widely-studied of all the influenza viral proteins as the three-dimensional structures of both these proteins have been solved.

The three-dimensional structure of the H3 hemagglutinin along with sequence information on large numbers of variants has allowed for the elucidation of the antigenic sites on the HA molecule (Webster et al., 1983, In Genetics of Influenza Virus, P. Palese and D. W. Kingsbury, eds., Springer-Verlag, Vienna, pp. 127-160). These sites fall into four discrete non-overlapping regions on the surface of the HA. These regions are highly variable and have also been shown to be able to accept insertions and deletions. Therefore, substitution of these sites within HA (e.g., site A; amino acids 122-147 of the A/HK/68 HA) with a portion of a foreign protein may provide for a vigorous humoral response against this foreign peptide. In a different approach, the foreign peptide sequence may be inserted within the antigenic site without deleting any viral sequences. Expression products of such constructs may be useful in vaccines against the foreign antigen, and may indeed circumvent a problem discussed earlier, that of propagation of the recombinant virus in the vaccinated host. An intact HA molecule with a substitution only in antigenic sites may allow for HA function and thus allow for the construction of a viable virus. Therefore, this virus can be grown without the need for additional helper functions. Of course, the virus should be attenuated in other ways to avoid any danger of accidental escape.

Other hybrid constructions may be made to express proteins on the cell surface or enable them to be released from the cell. As a surface glycoprotein, the HA has an amino-terminal cleavable signal sequence necessary for transport to the cell surface, and a carboxy-terminal sequence necessary for membrane anchoring. In order to express an intact foreign protein on the cell surface it may

be necessary to use these HA signals to create a hybrid protein. Alternatively, if only the transport signals are present and the membrane anchoring domain is absent, the protein may be excreted out of the cell.

In the case of the NA protein, the three-dimensional structure is known but the antigenic sites are spread out over the surface of the molecule and are overlapping. This indicates that if a sequence is inserted within the NA molecule and it is expressed on the outside surface of the NA it will be immunogenic. Additionally, as a surface glycoprotein, the NA exhibits two striking differences from the HA protein. Firstly, the NA does not contain a cleavable signal sequence; in fact, the amino-terminal signal sequence acts as a membrane anchoring domain. The consequence of this, and the second difference between the NA and HA, is that the NA is orientated with the amino-terminus in the membrane while the HA is orientated with the carboxy-terminus in the membrane. Therefore it may be advantageous in some cases to construct a hybrid NA protein, since the fusion protein will be orientated opposite of a HA-fusion hybrid.

5.1.6. INSERTION OF THE HETEROLOGOUS GENE INTO THE NS AND M GENE SEGMENTS

The unique property of the NS and M segments as compared to the other six gene segments of influenza virus is that these segments code for at least two protein products. In each case, one protein is coded for by an mRNA which is co-linear with genomic RNA while the other protein is coded for by a spliced message. However, since the splice donor site occurs within the coding region for the co-linear transcript, the NS1 and NS2 proteins have an identical 10 amino acid amino terminus while M1 and M2 have an idential 14 amino acid amino terminus.

As a result of this unique structure, recombinant viruses may be constructed so as to replace one gene product within the segment while leaving the second product intact. For instance, replacement of the bulk of the NS2 or M2 coding region with a foreign gene product (keeping the splice acceptor site) could result in the expression of an intact NS1 or M1 protein and a fusion protein instead of NS2 or M2. Alternatively, a foreign gene may be inserted within the NS gene segment without affecting either NS1 or NS2 expression. Although most NS genes contain a substantial overlap of NS1 and NS2 reading frames, certain natural NS genes do not. We have analyzed the NS gene segment from A/Ty/Or/71 virus (Norton et al., 1987, Virology 156:204-213) and found that in this particular gene, the NS1 protein terminates at nucleotide position 409 of the NS gene segment while the splice acceptor site for the NS2 is at nucleotide position 528. Therefore, a foreign gene could be placed between the termination codon of the NS1 coding region and the splice acceptor site of the NS2 coding region without affecting either protein. It may be necessary to include a splice acceptor site at the 5' end of the foreign gene sequence to ensure protein production (this would encode a hybrid protein containing the amino-terminus of NS1). In this way, the recombinant virus should not be defective and should be able to be propagated without need of helper functions.

Although the influenza virus genome consists of eight functional gene segments it is unknown how many actual segments a virus packages. It has been suggested that influenza can package more than eight segments, and possibly up to 12 (Lamb and Choppin, 1983, Ann. Rev. Biochem. 52:467-506). This would allow for easier propagation of recombinant virus in that "ninth" gene segment could be designed to express the foreign gene product. Although this "ninth" segment may be incorporated into some viruses, it would soon be lost during virus growth unless some selection is

supplied. This can be accomplished by "uncoupling" the NS or M gene segment. The NS2 coding portion could be removed from the NS gene segment and placed on the gene segment coding for the foreign protein (along with appropriate splicing signals). Alternatively, a bicistronic mRNA could be constructed to permit internal initiation to "unsplice" these viral sequences; for example, using the sequences described by Pelletier et al., 1988, Nature 334:320-325.

The resulting recombinant virus with the "uncoupled" NS or M gene would be able to propagate on its own and also would necessarily have to package the "ninth" gene segment, thus ensuring expression of the foreign gene.

5.2. EXPRESSION OF HETEROLOGOUS GENE PRODUCTS USING RECOMBINANT RNA TEMPLATE

The recombinant templates prepared as described above can be used in a variety of ways to express the heterologous gene products in appropriate host cells or to create chimeric viruses that express the heterologous gene products. In one embodiment, the recombinant template can be combined with viral polymerase complex purified as described in Section 6, infra, to produce rRNPs which are infectious. Alternatively, the recombinant template may be mixed with viral polymerase complex prepared using recombinant DNA methods (e.g. see Kingsbury et al., 1987, Virology 156:396-403). Such rRNPs, when used to transfect appropriate host cells, may direct the expression of the heterologous gene product at high levels. Host cell systems which provide for high levels of expression include continuous cell lines that supply viral functions such as cell lines superinfected with influenza, cell lines engineered to complement influenza viral functions, etc.

In an alternate embodiment of the invention, the recombinant templates or the rRNPs may be used to transfect cell lines that express the viral polymerase proteins in order to achieve expression of the heterologous gene product.

To this end, transformed cell lines that express all three polymerase proteins such as 3P-38 and 3P-133 (Krystal et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:2709-2713) may be utilized as appropriate host cells. Host cells may be similarly engineered to provide other viral functions or additional functions such as NP.

5.2.1. PURIFICATION OF THE VIRAL POLYMERASE

The viral polymerase proteins used to produce the rRNPs may be purified from dissociated RNP cores isolated from whole virus. In general, RNP cores may be prepared using standard methods (Plotch et al., 1981, Cell 23:847-858; Rochavansky, 1976, Virology 73:327-338). The pooled RNP cores may then be centrifuged on a second gradient of CsCl (1.5-3.0 M) and glycerol (30%-45%) as described by Honda et al., 1988, J. Biochem. 104:1021-1026. The active viral polymerase fractions may be isolated from top of the gradient, i.e. in the region of the gradient correlating with 1.5 to 2.0 M CsCl and corresponding to the fraction Honda et al. identified as "NP". Surprisingly, this fraction contains all the viral polymerase proteins required for the active complex. Moreover, the P proteins which may be recovered from the bottom of the gradient are not required, and indeed do not provide for the transcription of full length viral RNA. Thus, it appears that the so-called "NP" fraction contains, in addition to NP, the active forms of the PB2, PB1, and PA proteins.

5.2.2. HIGH CONCENTRATIONS OF POLYMERASE ARE REQUIRED FOR CAP-PRIMED RNA SYNTHESIS

High concentrations of viral polymerase complex are able to catalyze this virus-specific cap-endonuclease primed transcription. Under the conditions specified in Section 6 infra, about 50 ng NP with 200 pg of the three P proteins

were found to react optimally with 5 to 10 ng RNA reaction. The observation has been that although the NP selectively encapsidates influenza vRNA or cRNA in vivo, the NP will bind to RNA nonspecifically in vitro (Kingsbury, et al., 1987, Virology 156: 396-403; Scholtissek and Becht, 1971, J. Gen. Virol. 10: 11-16). Presumably, in order for the viral polymerase to recognize the viral template RNAs in our in vitro reaction, they have to be encapsidated by the NP. Therefore, the addition of a capped mRNA primer would essentially compete with the template RNA for binding of NP. Since the dinucleotide ApG would not be expected to bind NP, the low concentration polymerase was able to use only the short templates with ApG. Supporting this hypothesis is the observation that the higher concentration polymerase preparation is inhibited through the addition of progressively higher amounts of either template RNA or any non-specific RNA. It should also be noted that the unusual specificity for the m7GpppXm cap 1 structure previously shown with viral RNPs was also found with the reconstituted RNPs.

5.2.3. GENOMIC LENGTH RNA TEMPLATES ARE EFFICIENTLY COPIED

Plasmid-derived RNA identical to segment 8 of the A/WSN/33 virus was specifically copied by the polymerase (using the PCR method described in FIG. 10). In reactions using RNA extracted from virus, all eight segments were copied, although the HA gene was copied at a lower level. The background in these reactions was decreased in comparison to the 30 to 53 nt templates, probably since the contaminating RNAs in the polymerase preparation were predominantly defective RNAs of small size. Recombinant templates encoding foreign genes transcribed in this system may be used to rescue the engineered gene in a virus particle.

5.3. PREPARATION OF CHIMERIC NEGATIVE STRAND RNA VIRUS

In order to prepare chimeric virus, reconstituted RNPs containing modified influenza virus RNAs or RNA coding for foreign proteins may be used to transfect cells which are also infected with a "parent" influenza virus. Alternatively, the reconstituted RNP preparations may be mixed with the RNPs of wild type parent virus and used for transfection directly. Following reassortment, the novel viruses may be isolated and their genomes be identified through hybridization analysis. In additional approaches described herein for the production of infectious chimeric virus, rRNPs may be replicated in host cell systems that express the influenza viral polymerase proteins (e.g., in virus/host cell expression systems; transformed cell lines engineered to express the polymerase proteins, etc.), so that infectious chimeric virus are rescued; in this instance, helper virus need not be utilized since this function is provided by the viral polymerase proteins expressed. In a particularly desirable approach, cells infected with rRNPs engineered for all eight influenza virus segments may result in the production of infectious chimeric virus which contain the desired genotype; thus eliminating the need for a selection system.

Theoretically, one can replace any one of the eight gene segments, or part of any one of the eight segments with the foreign sequence. However, a necessary part of this equation is the ability to propagate the defective virus (defective because a normal viral gene product is missing or altered). A number of possible approaches exist to circumvent this problem. We have shown that mutants of influenza virus defective in the PB2 and NP proteins can be grown to substantially higher titers in cell lines which were constructed to constitutively express the polymerase and NP proteins (Krystal et al., 1986 Proc. Natl. Acad. Sci. U.S.A.

83:2709-2813). Similar techniques may be used to construct transformed cell lines that constitutively express any of the influenza genes. These cell lines which are made to express the viral protein may be used to complement the defect in the recombinant virus and thereby propagate it. Alternatively, certain natural host range systems may be available to propagate recombinant virus. An example of this approach concerns the natural influenza isolate CR43-3. This virus will grow normally when passaged in primary chick kidney cells (PCK) but will not grow in Madin-Darby canine kidney cells (MDCK), a natural host for influenza (Maassab & DeBorde, 1983, Virology 130:342-350). When we analyzed this virus we found that it codes for a defective NS1 protein caused by a deletion of 12 amino acids. The PCK cells contain some activity which either complements the defective NS1 protein or can completely substitute for the defective protein.

A third approach to propagating the recombinant virus may involve co-cultivation with wild-type virus. This could be done by simply taking recombinant virus and co-infecting cells with this and another wild-type virus (preferably a vaccine strain). The wild-type virus should complement for the defective virus gene product and allow growth of both the wild-type and recombinant virus. This would be an analagous situation to the propagation of defective-interfering particles of influenza virus (Nayak et al., 1983, In: Genetics of Influenza Viruses, P. Palese and D. W. Kingsbury, eds., Springer-Verlag, Vienna, pp. 255-279). In the case of defective-interfering viruses, conditions can be modified such that the majority of the propagated virus is the defective particle rather than the wild-type virus. Therefore this approach may be useful in generating high titer stocks of recombinant virus. However, these stocks would necessarily contain some wild-type virus.

Alternatively, synthetic RNPs may be replicated in cells co-infected with recombinant viruses that express the influenza virus polymerase proteins. In fact, this method may be used to rescue recombinant infectious virus in accordance with the invention. To this end, the influenza virus polymerase proteins may be expressed in any expression vector/host cell system, including but not limited to viral expression vectors (e.g., vaccinia virus, adenovirus, baculovirus, etc.) or cell lines that express the polymerase proteins (e.g., see Krystal et al., 1986, Proc. Natl. Acad. Sci. USA 83: 2709-2713). Moreover, infection of host cells with rRNPs encoding all eight influenza virus proteins may result in the production of infectious chimeric virus particles. This system would eliminate the need for a selection system, as all recombinant virus produced would be of the desired genotype. In the examples herein, we describ a completely synthetic replication system where, rather than infecting cells with influenza virus, synthetic RNP's are replicated in cells through the action of influenza virus proteins expressed by recombinant vaccinia vectors. In this way we show that the only influenza virus proteins essential for transcription and replication of RNP are the three polymerase proteins and the nucleoprotein.

It should be noted that it may be possible to construct a recombinant virus without altering virus viability. These altered viruses would then be growth competent and would not need helper functions to replicate. For example, alterations in the hemagglutinin gene segment and the NS gene segment discussed, supra, may be used to construct such viable chimeric viruses.

In the examples infra, the construction of a recombinant plasmid is described that, following transcription by T7 polymerase, yielded an RNA template which was recognized and transcribed by the influenza virus polymerase in vitro. This RNA template corresponds to the NS

RNA of an influenza virus except that the viral coding sequences are replaced by those of a CAT gene. This recombinant negative strand viral RNA template was then mixed with purified influenza virus polymerase to reconstitute an RNP complex. The recombinant RNP complex was transfected into cells which were then infected with influenza virus, leading to expression of CAT activity.

A number of factors indicate that this system represents a biologically active recombinant RNP complex which is under tight control of the signals for transcription, replication and packaging of influenza virus RNAs. First, the CAT gene is of negative polarity in the recombinant viral RNA used for RNP transfection. Thus, the incoming RNA cannot be translated directly in the cell and must first be transcribed by the influenza virus polymerase to permit translation and expression of the CAT gene. Secondly, neither transfected naked recombinant RNA alone in the presence of infecting helper virus, nor recombinant RNP complex in the absence of infecting helper virus is successful in inducing CAT activity. This suggests that influenza viral proteins provided by the incoming RNP, as well as by the infecting helper virus, are necessary for the amplification of the recombinant RNA template. Finally, after RNP-transfection and infection by helper virus, virus particles emerge which apparently contain the recombinant RNA, since these particles again induce CAT activity in freshly infected cells. These results suggest that the 26 3′ terminal and the 22 5′ terminal nucleotides corresponding to the terminal nucleotides in the influenza A virus NS RNA are sufficient to provide the signals for polymerase transcription and replication, as well as for packaging of the RNA into particles.

The foregoing results, which defined the cis acting sequences required for transciption, replication and packaging of influenza virus RNAs, were extended by

additional working examples, described infra, which demonstrate that recombinant DNA techniques cán be used to introduce site-specific mutations into the genomes of infectious influenza viruses.

Synthetic RNAs, derived by transcription of plasmid RNA in vitro were used in RNP-transfection experiments to rescue infectious influenza virus. To enable selection of this virus, we chose a system that required the presence of a WSN-like neuraminidase gene in the rescued virus. Viruses containing this gene can grow in MDBK cells in the absence of protease in the medium (Schulman et al., 1977, J. Virol. 24:170-176). The helper virus WSN-HK does not grow under these circumstances. Clearly, alternative selection systems exist. For example, antibody screens or conditionally lethal mutants could be used to isolate rescued viruses containing RNAs derived from plasmid DNAs. In the experiments viruses described infra, viruses which were WSN virus-like were recovered. The WSN NA gene was derived from plasmid DNAs or from purified WSN virion RNA (FIG. 17, lanes 2 and 5). In the latter case, using whole virion RNA for the RNP-transfection, we do not know whether other genes were also transfered to the rescued virus, since the helper virus shares the remaining seven genes with WSN virus. The rescued viruses had the expected RNA patterns (FIG. 17) and grew to titers in MDBK or MDCK cells which were indistinguishable from those of the wild type WSN virus. It should be noted that rescue of an NA RNA containing a single nucleotide deletion in the 5′ nontranslated region was not possible. This again illustrates the importance of regulatory sequences present in the non-translated regions of influenza virus RNAs. We also rescued virus using RNA that was engineered to contain 5 nucleotide changes in a 39 nucleotide long region (FIG. 16). We verified the presence of these mutations in the rescued mutant virus by direct sequencing of the RNA (FIG. 18). These mutations did not result in any amino acid

change in the neuraminidase protein and thus were not expected to change the biological property of the virus. Although this virus was not extensively studied, its plaquing behavior and its growth characteristics were indistinguishable from that of wild type WSN virus. Using such technology, mutations may be introduced that will change the biological characteristics of influenza viruses. These studies will help in distinguishing the precise functions of all the viral proteins, including those of the nonstructural proteins. In addition, the nontranslated regions of the genome can be studied by mutagenesis, which should lead to a better understanding of the regulatory signals present in viral RNAs. An additional area of great interest concerns the development of the influenza virus system as a vaccine vector.

5.4. VACCINE FORMULATIONS USING THE CHIMERIC VIRUSES

Virtually any heterologous gene sequence may be constructed into the chimeric viruses of the invention for use in vaccines. Preferably, epitopes that induce a protective immune response to any of a variety of pathogens, or antigens that bind neutralizing antibodies may be expressed by or as part of the chimeric viruses. For example, heterologous gene sequences that can be constructed into the chimeric viruses of the invention for use in vaccines include but are not limited to epitopes of human immunodeficiency virus (HIV) such as gp120; hepatitis B virus surface antigen (HBsAg); the glycoproteins of herpes virus (e.g. gD, gE); VP1 of poliovirus; antigenic determinants of non-viral pathogens such as bacteria and parasites, to name but a few. In another embodiment, all or portions of immunoglobulin genes may be expressed. For example, variable regions of anti-idiotypic immunoglobulins that mimic such epitopes may be constructed into the chimeric viruses of the invention.

Either a live recombinant viral vaccine or an inactiviated recombinant viral vaccine can be formulated. A live vaccine may be preferred because multiplication in the host leads to a prolonged stimulus of similar kind and magnitude to that occurring in natural infections, and therefore, confers substantial, long-lasting immunity. Production of such live recombinant virus vaccine formulations may be accomplished using conventional methods involving propagation of the virus in cell culture or in the allantois of the chick embryo followed by purification.

In this regard, the use of genetically engineered influenza virus (vectors) for vaccine purposes may require the presence of attenuation characteristics in these strains. Current live virus vaccine candidates for use in humans are either cold adapted, temperature sensitive, or passaged so that they derive several (six) genes from avian viruses, which results in attenuation. The introduction of appropriate mutations (e.g., deletions) into the templates used for transfection may provide the novel viruses with attenuation characteristics. For example, specific missense mutations which are associated with temperature sensitivity or cold adaption can be made into deletion mutations. These mutations should be more stable than the point mutations associated with cold or temperature sensitive mutants and reversion frequencies should be extremely low.

Alternatively, chimeric viruses with "suicide" characteristics may be constructed. Such viruses would go through only one or a few rounds of replication in the host. For example, cleavage of the HA is necessary to allow for reinitiation of replication. Therefore, changes in the HA cleavage site may produce a virus that replicates in an appropriate cell system but not in the human host. When used as a vaccine, the recombinant virus would go through a single replication cycle and induce a sufficient level of immune response but it would not go further in the human host and

cause disease. Recombinant viruses lacking one or more of the essential influenza virus genes would not be able to undergo successive rounds of replication. Such defective viruses can be produced by co-transfecting reconstituted RNPs lacking a specific gene(s) into cell lines which permanently express this gene(s). Viruses lacking an essential gene(s) will be replicated in these cell lines but when administered to the human host will not be able to complete a round of replication. Such preparations may transcribe and translate -- in this abortive cycle -- a sufficient number of genes to induce an immune response. Alternatively, larger quantities of the strains could be administered, so that these preparations serve as inactivated (killed) virus vaccines. For inactivated vaccines, it is preferred that the heterologous gene product be expressed as a viral component, so that the gene product is associated with the virion. The advantage of such preparations is that they contain native proteins and do not undergo inactivation by treatment with formalin or other agents used in the manufacturing of killed virus vaccines.

In another embodiment of this aspect of the invention, inactivated vaccine formulations may be prepared using conventional techniques to "kill" the chimeric viruses. Inactivated vaccines are "dead" in the sense that their infectivity has been destroyed. Ideally, the infectivity of the virus is destroyed without affecting its immunogenicity. In order to prepare inactivated vaccines, the chimeric virus may be grown in cell culture or in the allantois of the chick embryo, purified by zonal ultracentrifugation, inactivated by formaldehyde or β-propiolactone, and pooled. The resulting vaccine is usually inoculated intramuscularly.

Inactivated viruses may be formulated with a suitable adjuvant in order to enhance the immunological response. Such adjuvants may include but are not limited to mineral gels, e.g., aluminum hydroxide; surface active substances

such as lysolecithin, pluronic polyols, polyanions; peptides; oil emulsions; and potentially useful human adjuvants such as BCG and Corynebacterium parvum.

Many methods may be used to introduce the vaccine formulations described above, these include but are not limited to oral, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, and intranasal routes. It may be preferable to introduce the chimeric virus vaccine formulation via the natural route of infection of the pathogen for which the vaccine is designed. Where a live chimeric virus vaccine preparation is used, it may be preferable to introduce the formulation via the natural route of infection for influenza virus. The ability of influenza virus to induce a vigorous secretory and cellular immune response can be used advantageously. For example, infection of the respiratory tract by chimeric influenza viruses may induce a strong secretory immune response, for example in the urogenital system, with concomitant protection against a particular disease causing agent.

6. EXAMPLE: PROMOTER ANALYSIS OF THE INFLUENZA VIRAL RNA POLYMERASE

In the examples described below, polymerase which is depleted of genomic RNA was prepared from the upper fractions of the CsCl-glycerol gradient centrifugation. This polymerase is able to copy short model templates which are derived from transcription of appropriate plasmid DNA with bacteriophage T7 RNA polymerase in a sequence-specific manner. The termini of this model RNA are identical to the 3′ 15 and 5′ 22 nucleotides conserved in segment 8 from all influenza A viral RNAs. By manipulating the plasmid in order to prepare different RNAs to serve as template, we demonstrated that recognition of and synthesis from this model RNA was specific for the promoter at the 3′ terminal sequence and did not require the panhandle. In addition,

site specific mutagenesis identified nucleotide positions responsible for the viral polymerase favoring synthesis from genomic sense templates over complementary sense RNA. Conditions were also found in which cap-endonuclease primed RNA synthesis could be observed using model RNAs. In addition, the reconstituted system permitted virus-specific synthesis from genomic length RNAs, derived either from plasmids or from RNA purified from virus through phenol extraction.

6.1. MATERIALS AND METHODS

6.1.1. PURIFICATION OF THE VIRAL RNA POLYMERASE

RNP cores were prepared from whole virus using standard methods (Plotch, et al., 1981, Cell 23: 847-858; Rochavansky, 1976, Virology 73: 327-338). Two to three milligrams of virus were disrupted by incubating in 1.5% Triton N-101, 10 mg/ml lysolecithin, 100 mM tris-HCl, pH 8.0, 100 mM KCl, 5 mM $MgCl_2$, 5% glycerol and 1.5 mM dithiothreitol. The sample was fractionated by centrifugation on a 30-70% glycerol (w/v) step gradient in the presence of 50 mM tris-HCl, pH 7.8 and 150 mM NaCl. The core preparation was centrifuged at 45,000 rpm in an SW50.1 rotor for 4 hours at 4°C. Fractions enriched in RNP were identified by SDS-polyacrylamide gel electrophoresis of protein samples from each fraction and staining with silver. The core fractions were then subjected to a second gradient centrifugation as was described in Honda et al. 1988, J. Biochem. 104: 1021-1026. This second gradient had steps of 0.5 ml 3.0 M CsCl and 45% (w/v) glycerol, 1.75 ml 2.5 M CsCl and 40% glycerol, 1.25 ml 2.0 M CsCl and 35% glycerol, and 1.0 ml of 1.5 M CsCl and 30% glycerol. All steps were buffered with 50 mM tris-HCl, pH 7.6 and 100 mM NaCl. 0.5 ml of RNP cores were layered on top and the sample was centrifuged at 45,000 rpm in an SW50.1 rotor for 25 hours at 4°C. Polymerase fractions were again identified by SDS-

polyacrylamide electrophoresis of the protein samples and silver staining. Active polymerase fractions were generally found in the region of the gradient correlating with 1.5 to 2.0 M CsCl. These fractions were pooled and then dialyzed against 50 mM tri-HCl, pH 7.6, 100 mM NaCl and 10 mM $MgCl_2$ and concentrated in centricon-10 tubes (Amicon) or fractions were dialyzed in bags against 50 mM tris-HCl, pH 7.6, 100 mM NaCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, and 50% glycerol.

6.1.2. PREPARATION OF PLASMID

The plasmid design is indicated in FIG. 2. Insert DNA for the pV-wt plasmid was prepared using an Applied Biosystems DNA synthesizer. The "top" strand was 5'-GAAGCTTAATACGACTCACTATAAGTAGAAACAAGGGTGTTTTTTCATATCATTTAAACTTC ACCCTGCTTTTGCTGAATTCATTCTTCTGCAGG-3'. The "bottom" strand was synthesized by primer-extension with 5'-CCTGCAGAAGAATGA-3' as primer. The 95 bp DNA was digested with HindIII and PstI and purified by extraction with phenol/chloroform, ethanol precipitation, and passage over a NACS-prepack ion exchange column (Bethesda Research Laboratories). This DNA was ligated into pUC-19 which had been digested with HindIII and PstI and then used to transform E. coli strain DH5-α which had been made competent using standard protocols. Bacteria were spread on agar plates containing X-gal and IPTG, and blue colonies were found to have the plasmid containing the predicted insert since the small insert conserved the lacZ reading frame and did not contain a termination codon. The pM-wt plasmid was prepared by a similar strategy except that both strands were chemically synthesized with the upper strand having the sequence 5'-GAAGCTTAATACGACTCACTATAAGCAAAAGCAGGGTGAAGTTTAAATGATAT-GAAAAAACACCCTTGTTTCTACTGAATTCATTCTTCTGCAGG-3'.

The pV-d5' plasmid (FIG. 2) was prepared using the oligonucleotides 5'-AGCTTAATACGACTCACTATAAGATCTATTAAACT-TCACCCTGCTTTTGCTGAATTCATTCTTCTGCA-3' and 5'-GAAGAATGAAT-

TCAGCAAAAGCAGGGTGAAGTTTAATAGATCTTATAGTGAGTCGTATTA-3′. The DNAs were annealed and ligated into the HindIII/PstI digested pUC-19 and white colonies were found to contain the correct plasmid because this insert resulted in a frameshift in the lacZ gene. The point mutants were isolated following digestion of pV-d5′ with BglII and PstI and ligation of the linearized plasmid with a single stranded oligonucleotide of mixed composition. Since BglII laves a 5′ extension and PstI a 3′ extension, a single oligonucleotide was all that was necessary for ligation of insert. The host cell was then able to repair gaps caused by the lack of a complementary oligonucleotide. Oligonucleotides were designed to repair the frameshift in the lacZ gene so that bacteria which contained mutant plasmids were selected by their blue color.

Plasmid pHgaNS, which was used to prepare an RNA identical to segment 8 of A/WSN/33, was prepared using the primers 5′-CCGAATTCTTAATACGACTCACTATAAGTAGAAACAAGGGTG-3′ and 5′-CCTCTAGACGCTCGAGAGCAAAAGCAGGTG-3′ in a polymerase chain reaction off a cDNA clone. The product was then cloned into the XbaI/EcoRI window of pUC19.

6.1.3. PREPARATION OF RNA TEMPLATES

Plasmid DNAs were digested with MboII or other appropriate endonucleases (see FIG. 2), and the linearized DNA was transcribed using the bacteriophage T7 RNA polymerase. Run-off RNA transcripts were treated with RNAse-free DNAse 1 and then the RNA was purified from the proteins and free nucleotides using Qiagen tip-5 ion exchange columns (Qiagen, Inc.). Following precipitation in ethanol, purified RNAs were resuspended in water and a sample was analyzed by electrophoresis and followed by silver staining of the polyacrylamide gel in order to quantitate the yield of RNA.

6.1.4. INFLUENZA VIRAL POLYMERASE REACTIONS

In a 25 μl total volume, about 30 μg of nucleoprotein and 200 pg total of the three polymerase proteins were mixed with 10 ng of template RNA and the solution was made up to a final concentration of: 50 mM Hepes pH 7.9, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, 0.05% NP-40, 0.4 mM adenylyl-(3'-5')-guanosyl (ApG) dinucleotide (Pharmacia), 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP and approximately 0.6 μM α-^{32}P-UTP (40 μCi at 3000 Ci/mmole, New England Nuclear). Reactions were assembled on ice and then transferred to a 30°C water bath for 90 minutes. Reactions were terminated by the addition of 0.18 ml ice-cold 0.3 M sodium acetate/10 mM EDTA and were then extracted with phenol/chloroform (1:1 volume ratio). Following the first extraction, 15 μg polyI-polyC RNA was added as carrier, and the sample was extracted again with phenol/chloroform. The samples were then extracted with ether and precipitated in ethanol. Following centrifugation, the RNA pellet was washed twice with 70% ethanol and then dried under vacuum.

In reactions using the high concentration polymerase, conditions were identical as above except that 20 ng of template RNA were added. In reactions using genomic length RNAs, the amount of polymerase used was doubled, 50 ng of template RNA was used, and the UTP concentration was raised to 2.6 μM.

The RNA was resuspended in a dye mix containing 78% formamide, 10 mM EDTA, 0.1% xylene cyanol and 0.05% bromophenol blue. Typically, a sample from this RNA was electrophoresed on an 8% polyacrylamide gel in the absence of urea, and the remainder was denatured by heating to 100°C for 1.5 minutes and an aliquot was loaded on an 8% polyacrylamide gel containing 7.7 M urea. Gels were fixed by a two step procedure, first in 10% acetic acid, and then in 25% methanol/8% acetic acid. Gels were dried onto filter paper and then exposed to x-ray film.

When different RNAs were being tested for use as template, the different RNA preparations were always analyzed on polyacrylamide gels and stained with silver in order that equal amounts of each template were used. To quantitate the amount of product, gels were exposed to x-ray film in the absence of an intensifying screen in order to improve the linearity of the densitometer readings. Autoradiographs were analyzed using a FB910 scanning densitometer (Fisher Biotech) and peaks were evaluated using computer software from Fisher Biotech.

6.1.5. NUCLEASE ANALYSIS OF REACTION PRODUCTS

For ribonuclease T1 analysis of the two principle RNA products, reaction products were analyzed by 8% polyacrylamide gel electrophoresis (without urea) and the gel was not treated with fixative. The wet gel was exposed to an x-ray film and the appropriate gel pieces were located and excised. The gel piece was crushed in 0.3 ml containing 10 mM tris pH 7.5, 1 mM EDTA, 0.1% sodium dodecyl sulfate, and 1 µg tRNA as carrier. The RNA diffused into this solution for 3 hours and then the gel was pelleted and the supernatant was made 0.3M in sodium acetate. The supernatant was then extracted twice in phenol/chloroform and once in ether and then precipitated in ethanol. The RNA pellet was resuspended in 5 µl formamide, denatured in boiling water for 1.5 minutes and then diluted by the addition of 0.1 ml 10 mM tris-HCl, pH 7.5, and 1 mM EDTA. Ribonuclease T1 (50 units, Boehringer Mannheim Biochemicals) was added and the samples were incubated for 60 minutes at 37°C. V-wt and M-wt RNAs synthesized with T7 RNA polymerase in the presence of α-^{32}P-UTP were similarly digested with RNAse T1. Reaction products were extracted in phenol/chloroform and precipitated in ethanol and then were analyzed on 20% polyacrylamide gels containing 7.7 M urea.

Nuclease S1 analysis of reaction products was done on transcribed RNA by first terminating the standard polymerase reaction through the addition of S1 buffer to a volume of 0.2 ml with 0.26 M NaCl, 0.05 M sodium acetate, pH 4.6, and 4.5 mM zinc sulfate. The sample was divided into two 0.1 ml volumes and 100 units of S1 nuclease (Sigma Chemical Company) were added to one tube. The samples were incubated for 60 minutes at 37°C. Following the incubation, EDTA (10 mM final concentration) and 15 μg polyI-polyC RNA was added and the sample was extracted with phenol/chloroform and precipitated in ethanol. The samples were then subjected to polyacrylamide gel electrophoresis.

6.2. RESULTS

6.2.1. PREPARATION OF INFLUENZA VIRAL RNA POLYMERASE AND OF TEMPLATE RNA

RNP cores of influenza virus A/Puerto Rico/8/34 were prepared by disruption of virus in lysolecithin and Triton N-101 followed by glycerol gradient centrifugation (Rochavansky, 1976, Virology 73: 327-338). Fractions containing cores were then subjected to a second centrifugation in a CsCl-glycerol step gradient (Honda, et al., 1988, J. Biochem. 104: 1021-1026). Fractions containing the polymerase were identified by gel electrophoresis of samples followed by silver-staining. FIG. 1 shows the polymerase preparation after CsCl centrifugation. Bovine serum albumin (BSA) was added during dialysis to protect against protein loss. Densitometric scanning of lane 4 compared to known quantities of whole virus in lanes 1 and 2 allowed us to estimate that the proteins in lane 4 consist of 150 ng of NP and about 1 ng total of the three polymerase proteins. One fifth of the preparation used for this gel was used per reaction.

The overall design of the plasmids used to prepare template RNAs in this study is depicted in Figure 2. The entire insert was prepared using oligonucleotides from a DNA synthesizer which were then cloned into the polylinker of pUC19. The insert contained a truncated promoter sequence recognized by the bacteriophage T7 RNA polymerase (Studier and Dunn, 1983, Cold Spring Harbor Symposia on Quantitative Biology, XLVII, 999-1007) so that the first nucleotides synthesized were the terminal 22 nucleotides (nt) of the conserved sequence from the 5′ end of the genome RNA. When the plasmid was cut with restriction endonuclease MboII (which cuts 7 bases upstream of its recognition site), the RNA which resulted from T7 RNA polymerase transcription ended with the terminal 3′ nucleotides of the influenza viral sequence. Included in the sequence was the poly-U stretch adjacent to the 5′ end of the conserved terminus which is thought to comprise at least part of the termination-polyadenylation signal (Robertson, et al., 1981, J. Virol. 38, 157-163). The total length of this model genomic RNA was 53 nt since a 16 nt spacer separated the terminal conserved sequences. The model RNA which contained both termini identical to those of vRNA was named V-wt. The RNA M-wt encoded the exact complementary strand of V-wt so that the termini match those of complementary RNA (cRNA). V-wt and M-wt were constructed to serve as models for influenza virus-specific vRNA and cRNA, respectively.

6.2.2. VIRAL POLYMERASE CATALYZES SYNTHESIS OF A FULL LENGTH COPY OF THE TEMPLATE

In the reaction using the influenza viral polymerase, V-wt template and ApG primer, a product was obtained which comigrated with a 53 nt RNA on denaturing gels. RNA migrating as a doublet at a position of about 40 to 45 nucleotides (FIG. 3A, lane 2) was also seen. This shorter product is shown below to be RNA which had terminated at a

stretch of adenosines present between nucleotides 43-48 in the virion sense template. In addition to the template specific transcripts, a general background of light bands could be seen which correspond to truncated RNA products transcribed from viral genomic RNA not removed during the CsCl-glycerol centrifugation step. When no primer is used, there was no specific transcription product seen (FIG. 3A, lane 3). Additional experiments showed globin mRNA, containing a terminal cap 1 structure, was inactive as primer using initial preparations of polymerase.

When the polymerase reaction was terminated by the addition of excess buffer favorable for nuclease S1 digestion and nuclease was added, the radioactively-labeled product was resistant to digestion (FIG. 3B, lane 2). By contrast these conditions very efficiently digested the V-wt single-stranded RNA radioactively synthesized with T7 RNA polymerase (FIG. 3B, lanes 3 and 4). These nuclease S1 data confirmed that the opposite strand was indeed being synthesized in these reactions. The product of the reaction might be a double stranded RNA, but it could not be ruled out that the product was in fact single stranded and later annealed to the template RNA in the presence of high salt used in the nuclease reaction.

The RNA products were purified by electrophoresis on an 8% gel, excised, eluted from the gel, and then digested by ribonuclease T1. Products were analyzed by electrophoresis and compared to the patterns generated by RNase T1 digestion of internally labeled M-wt and V-wt control probes. As can be seen in FIG. 3C, the full length RNA (lane 1) has the identical pattern as does the plus sense RNA, M-wt (lane 3), and it does not have the pattern of the V-wt RNA (lane 4). The observed patterns were essentially identical to that which is predicted from the sequence of the RNA and thus showed that the polymerase faithfully copied the V-wt template. The smaller RNA product, a doublet with most

templates, was also digested with RNase T1. Its pattern was similar to that of the full length RNA product (FIG. 3C, lane 2) except the 14 base oligonucleotide was not present. Instead, a faint 13 base oligonucleotide was seen, thus mapping the termination of the short RNA to position 44, a site where two uridines would be incorporated. Since the amount of smaller RNA product decreased at higher UTP concentrations and disappeared when CTP was used as label, these bands appeared to be an artifact of low UTP concentrations in the polymerase reaction.

6.2.3. CONDITIONS FOR THE POLYMERASE REACTIONS USING MODEL RNA TEMPLATES

It was found that protein samples containing about 30 ng of NP protein and about 200 pg total of the three P proteins would react optimally with 5 to 10 ng of RNA. By using cold competitor RNA, polyI-polyC, it was found that excess RNA nonspecifically inhibited transcription, possibly via non-specific binding of the NP protein (Kingsbury, et al., 1987, Virology 156: 396-403; Scholtissek and Becht, 1971, J. Gen. Virol. 10: 11-16). In the absence of nonspecific competitor, variations in the amount of template between 1 and 10 ng produced little change in the efficiency of RNA synthesis. The NP protein and RNA were present at about equal molar concentrations and these were each about a thousand-fold in excess of the moles of the complex (assuming it to be 1:1:1) formed by the three P proteins in the typical reaction.

Since these reconstituted RNPs were able to use ApG but not globin mRNA as primer, we tested these model RNPs for other variables of the transcription reaction. In all other ways tested, the reconstituted RNPs behaved in solution similarly to those RNPs purified from detergent disrupted virus. The optimum temperature for RNA synthesis was 30°C (FIG. 4A, lane 2) as has been repeatedly found for the viral

polymerase (Bishop, et al., 1971, J. Virol. 8: 66-73; Takeuchi, et al., 1987, J. Biochem. 101: 837-845; Ulmanen, et al., 1983, J. Virol. 45: 27-35). Also, the most active salt conditions were 60 mM NaCl (FIG. 4B, lane 2), again consistent with conditions used by several groups (Bishop, et al., 1971, J. Virol. 8: 66-73; Honda, et al., 1988, J. Biochem. 104: 1021-1026; Shapiro, and Krug, 1988, J. Virol. 62: 2285-2290). Figure 4C shows a time-course experiment. The amount of RNA synthesis appeared to increase roughly linearly for the first 90 minutes, as was found for viral RNPs (Takeguchi, et al., 1987, J. Biochem. 101: 837-845).

6.2.4. SPECIFICITY OF THE ELONGATION REACTION

Various RNAs were tested for suitability as templates for the RNA polymerase of influenza virus. The pV-wt plasmid clone was digested with either EcoRI, PstI or SmaI, and T7 polymerase was used to transcribe RNA. This resulted in RNAs identical to V-wt except for the addition of 5, 13 and 38 nt at the 3′ end. In FIG. 5A an overexposure of an autoradiograph is shown in order to demonstrate that no transcripts over background were observed in reactions which contained as template: two of the RNAs identical to V-wt except they contained 13 and 38 nt of extra sequence on the 3′ terminus (lanes 1 and 2); a single stranded DNA of identical sequence to that of V-wt (lane 4); and an unrelated 80 nt RNA generated by transcribing the polylinker of pIBI-31 with T3 RNA polymerase (lane 5). However, the V-Eco template, containing five extra nucleotides on the 3′ end, could be recognized and faithfully transcribed, although at approximately one-third the efficiency of the wild type V-wt RNA (FIG. 5B, lane 3). It is interesting to note that initiation on the V-Eco RNA by the influenza viral polymerase appeared to occur at the correct base since the transcribed RNA was the same size as the product form the V-wt template.

6.2.5. ANALYSIS OF THE PROMOTER REGION FOR THE VIRAL RNA POLYMERASE

The original construct used for these studies contained the sequences of both RNA termini of genomic RNAs which could base pair and thus form a panhandle. This was done since it was shown that the vRNA in virions and in RNPs in infected cells was in circular conformation via the 15 to 16 nt long panhandle (Honda, et al., 1988, J. Biochem. 104: 1021-1026; Hsu, et al., 1987, Proc. Natl. Acad. Sci. USA 84: 8140-8144). It was further shown that the viral polymerase was bound to the double stranded structure (Honda, et al., 1988, J. Biochem. 104: 1021-1026), thus leading to the suggestion that the promoter for RNA synthesis was the panhandle. In order to test whether the panhandle was an absolute requirement for recognition, the following templates were used: the plasmid pV-wt was digested with DraI prior to transcription by the T7 polymerase (FIG. 2). This should result in an RNA molecule of 32 nt containing only virus-specific sequences from the 5′ end of the RNA. When this RNA was used as template, no apparent product was produced (FIG. 5B, lane 2). Therefore the 3′ terminus of virion RNA was required for this reaction. This finding was consistent with the fact that the initiation site at the 3′ end of V-wt was not present in V-Dra. A second plasmid clone was produced which deleted the 5′ terminal sequences but kept intact the 3′ terminus. This clone, pV-d5′, when digested with MboII and used for transcription by T7 polymerase produced a major transcript of 30 nt and minor species of 29 and 31 nt. Surprisingly, this template was recognized and copied by the influenza viral polymerase. FIG. 7, lane 1, shows that the product of the viral RNA polymerase reaction with V-d5′ contains multiple bands reflecting the input RNA. When the products shown in FIG. 7, lane 1, were eluted from gels and subjected to RNase T1 analysis, the pattern expected of the

transcription product of V-d5′ was observed. Since the V-d5′ RNA template was copied, the panhandle was not required for viral polymerase binding and synthesis.

Although the 5′ terminus was not required for synthesis by the polymerase, a distinct possibility was that V-wt RNA might be a preferred template as compared to V-d5′. In order to examine this, reactions were done in which the templates were mixed. The V-wt RNA was present at 5 ng in each reaction. The V-d5′ was absent (FIG. 6, lane 1) or was present at a 1/5 molar ratio (FIG. 6, lane 2) or a 1/1 molar ratio (FIG. 6, lane 3). The relative intensities of the bands from each RNA were determined by densitometry of the autoradiograph. The values were corrected for the amount of the radioactive nucleotide, UTP, which could be incorporated into each product, and the value was normalized so that the level of synthesis in each lane was set equal to one. The level of copying of V-wt decreased as V-d5′ was increased. When V-d5′ was present in one fifth molar ratio, its corrected level of synthesis was about one fourth of that from V-wt (FIG. 6, lane 2). When the two templates were present in equimolar amounts, the level of synthesis from V-wt was about 60% of the total (FIG. 6, lane 3) which might be within the expected range of experimental error for equivalent levels of synthesis. Similar results were obtained when V-d5′ RNA was kept constant and the V-wt RNA was varied. It was thus concluded that the panhandle-containing V-wt RNA was not greatly favored over the template RNA which only contained the proper 3′ terminus.

6.2.6. THE VIRAL POLYMERASE DOES NOT COPY RNA TEMPLATES CONTAINING PLUS-SENSE TERMINI

As described earlier, the influenza RNA polymerase performs three distinct activities during the course of an infection. Two activities involve the transcription of genome sense RNA and the third involves copying of the

complementary sense RNA into vRNA. We therefore constructed an RNA template which contained the 5′ and 3′ termini of the complementary sense RNA of segment 8 (M-wt; FIG. 2).

When the M-wt RNA was used as template, little synthesis was observed (FIG. 5B, lane 4). In two experiments used for quantitation, the average level of synthesis from M-wt RNA was 4% that of V-wt. In comparing the V-wt and M-wt RNA promoters, the M-wt has only three transition changes and one point insertion within the 3′ 15 nucleotides. These include a G to A change at position 3, a U to C change at position 5, a C to U change at position 8 and an inserted U between the ninth and tenth nucleotides (see Table II, below). In order to determine which of the four point differences in the 3′ termini were responsible for the specificity, many combinations of these were prepared and assayed for efficiency as a template (FIG. 7). These templates were derivatives of V-d5′ since they did not contain the 5′ terminus. The results of densitometry scans of several experiments are outlined in Table II.

TABLE II

QUANTITATIVE COMPARISON OF THE EFFECT OF POINT MUTATIONS IN THE PROMOTER SEQUENCE*

Template	3′ sequence	Level of RNA Synthesis
V-d5′	CACCCUGCUUUUGCU-OH	1
V-A3	CACCCUGCUUUUACU-OH	0.4
V-C5	CACCCUGCUUCUGCU-OH	1.0
V-$dU_{25}U_8$	CACCCUGUUUUUGCU-OH	1.0
V-U_8A_3	CACCCUGUUUUUACU-OH	0.08
V-U_8C_5	CACCCUGUUUCUGCU-OH	0.3
V-iU_{10}	CACCCUUGCUUUUGCU-OH	0.7
V-$iU_{10}A_3$	CACCCUUGCUUUUACU-OH	0.06
V-$iU_{10}U_8A_3$	CACCCUUGUUUUUACU-OH	0.2
V-$iU_{10}U_8C_5A_3$	CACCCUUGUUUCUACU-OH	0.2

* Sequences of V-wt, M-wt and V-d5′ are shown in FIG. 2. All other RNAs are identical to V-d5′ except for the indicated positions. The subscripted number indicates the distance from the 3′ end of a change, and d and i refer to deleted or inserted nucleotides.

As shown in Table II, single point changes in V-d5′ were equally well copied as compared to V-d5′ itself, except for the V-A_3 RNA which was copied at 40% efficiency (FIG. 7, lane 10; Table II). When RNAs with two changes were tested, the activity generally dropped to very low levels (FIG. 7, lanes 3, 4, and 5). Therefore, these experiments confirmed that the specificity of the reactions for V-wt over M-wt was the result of the combination of the nucleotide changes present at the 3′ terminus of M-wt.

6.2.7. CAP-ENDONUCLEASE PRIMED RNA SYNTHESIS

The method of purifying the viral polymerase was modified in order to decrease loss of protein during dialysis. Rather than using the Amicon centricon-10 dialysis system, the enzyme was dialyzed in standard membranes resulting in higher concentrations of all four viral core proteins. The pattern of the protein gel of this preparation was identical to that shown in FIG. 1, lane 4, except that there is no BSA-derived band. It was found that 5 μl of this preparation, containing 150 ng of NP and 5 ng total of the three polymerase proteins, reacted optimally with 10 to 40 ng of model RNA template. However, the use of higher levels of protein increased the background, possibly due to higher levels of contaminating RNAs (virion RNAs not removed by CsCl centrifugation) yielding products of the size class around 50-75 nt, complicating analysis of RNA templates containing a length of 50 nt.

This high concentration polymerase preparation was now active in cap-endonuclease primed RNA synthesis (FIG. 8A, lane 4) and also in primer-independent replication of the template RNA (FIG. 8A, lane 2). When globin mRNA was used as primer for transcription from the 30 nt V-d5′ template, a triplet of bands of size about 42 to 44 nt was apparent as product (FIG. 8A, lane 4), consistent with cleavage of the

cap structure at about 12 nt from the 5′ end of the mRNA and use of this oligonucleotide to initiate synthesis from the 30 nt model template. Since excess RNA inhibits RNA synthesis, probably via nonspecific binding of NP in vitro as discussed above, the optimal amount of cap donor RNA added to each reaction was found to be 100 ng, which is much lower than is usually used with preformed RNP structures (e.g. Bouloy, et al., 1980, Proc. Natl. Acad. Sci. USA 77:3952-3956). The most effective primer was ApG (FIG. 8A, lane 5 and lighter exposure in lane 6). The product migrates slower than that of the input template (FIG. 8A, lane 1) or the product in the absence of primer (FIG. 8A, lane 2) probably since the 5′ terminus of the ApG product is unphosphorylated. The intensity of the ApG-primed product was about ten-fold higher than that of the cap-primed product, but at 0.4 mM, ApG was at a 60,000-fold molar excess of the concentration of the cap donors. Thus, although the intensity of the product band from cap-priming was about ten-fold lower than that from ApG priming, the cap-primed reaction was about 6000-fold more efficient on a molar basis. This value is similar to the approximately 4000-fold excess efficiency observed previously for the viral polymerase (Bouloy, et al., 1980, Proc. Natl. Acad. Sci. USA 77: 3952-3956). It has been previously shown that cap donor RNAs containing a cap 0 structure, as in BMV RNA, are about ten-fold less active in priming the influenza viral polymerase (Bouloy, et al., 1980, Proc. Natl. Acad. Sci. USA 77: 3952-3956). This unusual cap specificity was shared by the reconstituted RNPs studied here as the specific product from the model RNA was greatly decreased in reactions containing BMV RNA as cap donor. A 30 nt product was observed in lanes 2-4, probably due to primerless replication of the model template.

That the product RNAs were of the opposite sense of the input template V-d5′ was shown by nuclease S1 analysis (FIG. 8B). The ApG-primed (FIG. 8B, lanes 1 and 2) and the

primerless (FIG. 8B, lanes 3 and 4) RNA products were essentially nuclease resistant. The product of the cap-primed reaction (FIG. 8B, lanes 5 and 6) was partially sensitive to nuclease as about 12 nt were digested from the product. These results were most consistent with the 5′ 12 nt being of mRNA origin as has been shown many times for influenza virus-specific mRNA synthesis.

The promoter specificity of this polymerase preparation in reactions primed with ApG was found to be essentially identical to those for the lower concentration enzyme as shown earlier. However, attempts thus far to perform similar analyses of promoter specificity with the primerless and cap-primed reactions have been frustrated by the comparatively high levels of background, thus making quantitation difficult.

6.2.8. REPLICATION OF GENOMIC LENGTH RNA TEMPLATES

A full length 890 nt RNA identical to the sequence of A/WSN/33 segment 8 was prepared by T7 RNA polymerase transcription of plasmid DNA, pHgaNS, which had been digested with restriction endonuclease HgaI. This RNA was copied in ApG-primed reactions containing 10 μl of the high concentration polymerase (FIG. 9, lane 8). That the RNA was in fact a copy of the template was demonstrated by its resistance to nuclease S1 (FIG. 9, lane 9). A similar product was observed in the absence of primer (FIG. 9, lanes 2 and 3). Confirmation that these product RNAs were full length copies of the template was done by RNase T1 analysis. Virion RNA purified from phenol-extracted A/PR/8/34 virus was similarly copied in ApG primed reaction (FIG. 9, lanes 10 and 11) and in the absence of primer (FIG. 9, lanes 4 and 5). Interestingly, the product from replication of the HA gene was at greatly reduced levels. The 3′ end of this RNA differs from that of segment 8 only at nucleotides 14 and 15, suggesting importance for these nucleotides in the promoter

for RNA synthesis. In addition, we found that when whole viral RNA was used in the reconstituted RNPs, the level of acid precipitable counts was about 70% of that observed with native RNPs. The viral polymerase was also able to copy these full length RNAs when globin mRNA was used in cap-primed reaction.

7. EXAMPLE: EXPRESSION AND PACKAGING OF A FOREIGN GENE BY RECOMBINANT INFLUENZA VIRUS

The expression of the chloramphenicol transferase gene (CAT) using rRNPs is described. The rRNPs were prepared using pIVACAT (originally referred to as pCATcNS), a recombinant plasmid containing the CAT gene. The pIVACAT plasmid is a pUC19 plasmid conaining in sequence: the T7-promoter; the 5'- (viral-sense) noncoding flanking sequence of the influenza A/PR8/34 RNA segment 8 (encodes the NS proteins); a BglII cloning site; the complete coding sequence of the chloramphenicol transferase (CAT) gene in the reversed and complemented order; the 3'- (viral-sense) noncoding NS RNA sequence; and several restriction sites allowing run-off transcription of the template. The pIVACAT can be transcribed using T7 polymerase to create an RNA with influenza A viral-sense flanking sequences around a CAT gene in reversed orientation.

The in vivo experiments described in the subsections below utilized the recombinant RNA molecule described containing sequences corresponding to the untranslated 3' and 5' terminal sequences of the NS RNA of influenza virus A/PR/8/34 flanking the antisense-oriented open reading frame of the CAT gene. This RNA was mixed with purified influenza virus polymerase complex and transfected into MDCK (or 293) cells. Following infection with influenza A/WSN/33 virus, CAT activity was measured in the RNP-transfected cells and amplification of the gene was indicated. In addition, the

recombinant influenza virus gene was packaged into virus particles, since CAT activity was demonstrated in cells following infection with the recombinant virus preparation.

7.1. MATERIALS AND METHODS

In order to get the flanking sequences of the NS RNA fused to the coding sequence of the CAT gene, the following strategy was used. Two suitable internal restriction sites were selected, close to the start and stop codon of the CAT gene, that would allow the replacement of the sequences flanking the CAT gene in the pCM7 plasmid with the 3′- and 5′- NS RNA sequences. At the 5′ and, a SfaNI site was chosen, (which generates a cut 57 nt from the ATG) and at the 3′- end a ScaI site which generates a cut 28 nt from the end of the gene (stop codon included). Next, four synthetic oligonucleotides were made using an Applied Biosystems DNA synthesizer, to generate two double-stranded DNA fragments with correct overhangs for cloning. Around the start codon these oligonucleotides formed a piece of DNA containing a XbaI overhang followed by a HgaI site and a PstI site, the 3′- (viral-sense) NS sequence immediately followed by the CAT sequence from start codon up to the SfaNI overhang (underscored). In addition a silent mutation was incorporated to generate an AccI site closer to the start codon to permit future modifications.

Xba I

Hga I Pst I Acc

5'-ctagacgccctgcagcaaaagcagggtgacaaagacataatggagaaaaaaatcac

3'tgcgggacgtcgttttcgtcccactgtttctgtattacctctttttttagtg

I SfaN I

tgggtataccaccgttgatatatcccaatcgcatcgtaaa- 3' oligo2

acccatatggtggcaactatatagggttagcgtagcatttcttg- 5' oligo1

Around the stop codon the two other oligonucleotides generated a piece of DNA as follows: a blunt-ended ScaI site, the CAT sequence from this site up to and including the stop codon (underlined) followed by a BglII site and a Xba I overhang.

Sca I Bgl II

5'-actgcgatgagtggcagggcggggcgtaatagat- 3' oligo3

3'-tgacgctactcaccgtcccgccccgcattatctagatc- 5' oligo4

XbaI

Using a single internal EcoRI site in the CAT sequence, the SfaNI/EcoRI and the EcoRI/ScaI fragment from pCM7 were independently cut out and purified from acrylamide gels. The SfaNI/EcoRI fragment was subsequently ligated with the synthetic DNA fragment obtained by annealing oligonucleotides 1 and 2 into a pUC19 plasmid that was cut with XbaI and EcoRI. The EcoRI/ScaI fragment was similarly cloned into an XbaI and EcoRI -digested pUC19 plasmid using oligonucleotides 3 and 4. The ligated DNA was transformed into competent DH5a bacteria, amplified, isolated and screened by means of restriction analysis using standard techniques.

The recombinants with the SfaNI containing insert were cut with XbaI and EcoRI and the plasmids with the ScaI insert were cut with EcoRI and BglII. The fragments were purified from acrylamide gel and cloned together into the pPHV vector which had been cut with XbaI and BglII. After transformation, white colonies were grown, analysed by endonuclease digestion and selected clones were sequenced. The final clone, pCATcNS2, was grown in large amounts and sequenced from the flanking pUC sequences up to 300 nt into the CAT gene, revealing no discrepancies with the intended sequence, with the exception of a G to A transition in the CAT gene, which appeared silent.

7.1.1. VIRUSES AND CELLS

Influenza A/PR/8/34 and A/WSN/33 viruses were grown in embryonated eggs and MDCK cells, respectively (Ritchey et al. 1976, J. Virol. 18: 736-744; Sugiura et al., 1972, J. Virol. 10: 639-647). RNP-transfections were performed on human 293 cells (Graham et al., 1977, J. Gen. Virol. 36:59-72) and on Madin-Darby canine kidney (MDCK) cells (Sugiura et al., 1972, supra).

7.1.2. CONSTRUCTION OF PLASMIDS

Plasmid pIVACAT1, derived from pUC19, contains the coding region of the chloramphenicol acetyltransferase (CAT) gene flanked by the noncoding sequences of the influenza A/PR/8/34 RNA segment 8. This construct is placed under the control of the T7 polymerase promoter in such a way that the RNA transcript IVACAT1 contains in 5′ to 3′ order: 22 nucleotides derived from the 5′ terminus of the influenza virus NS RNA, an 8 nt linker sequence including a BglII restriction site, the CAT gene in negative polarity, and 26 nt derived from the 3′ end of the influenza virus NS RNA (FIG. 11).

pIVACAT1 was constructed in the following way: In order to obtain the correct 5′- end in pIVACAT1, the EcoRI-ScaI fragment of the CAT gene derived from plasmid pCM7 (Pharmacia) was ligated to a DNA fragment formed by two synthetic oligonucleotides. The sequence of these oligonucleotides are: 5′-ACTGCGATGAGTGGCAGGGCGGGGCGTAATA-GAT- 3′ (top strand), and 5′-CTAGATCTATTACGCCCCGCCCTGCCAC-TCATCGCAGT- 3′ (bottom strand). For the 3′- end of the insert in pIVACAT1 the SfaN 1-EcoRI fragment of the CAT gene was ligated to a DNA fragment made up of the synthetic oligonucleotdies: 5′-CTAGACGCCCTGCAGCAAAAGCAGGGTGAC-AAAGACATAATGGAGAAAAAAAATCACTGGGTATACCACCGTTGATATATCCCAATCG-CATCGTAAA- 3′ (top strand), and 5′-GTTCTTTACGATGCGATTGGGAT-ATATCAACGGTGGTATACCCAGTGATTTTTTTCTCCATTATGTCTTTGTCACCCTGCT-TTTGCTGCAGGGCGT- 3′ (bottom strand). Oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer. These 5′ and 3′ constructs were ligated into pUC19 shuttle vectors digested with XbaI and EcoRI, grown up, cut out with EcoRI/BglII (5′ region) and XbaI/EcoRI (3′ region) and ligated into BglII/XbaI cut pPHV. The latter plasmid is similar to pV-WT described in Section 6, supra, except that it contains a BglII site which separates the noncoding terminal sequences of the influenza A virus NS RNA segment. The final clone pIVACAT1 (FIG. 1) was grown up and the DNA was partially sequenced starting from the flanking pUC sequences and reaching into the CAT gene. No changes were found as compared to the expected sequences with the exception of a silent G to A transition in the CAT gene at position 106 relative to the start of the IVACAT1 RNA.

7.1.3. T7 RNA TRANSCRIPTION

Plasmid pIVACAT1 was digested with HgaI (FIG. II), to allow run-off transcription. The 5 nt overhang generated by this enzyme was filled in with Klenow enzyme (BRL) and the DNA was purified over a spin column (Boehringer). The T7

polymerase reaction was performed using standard procedures in the presence of Rnasin (Promega). Template DNA was removed from Rnase free Dnase I (Promega). The RNA was purified over Qiagen tip-5 columns (Qiagen, Inc.) and quantitated using 4% polyacrylamide gels which were silver stained. NS RNA was prepared from plasmid pHgaNS in the same way.

7.1.4. PURIFICATION OF INFLUENZA A VIRUS POLYMERASE AND IN VITRO TRANSCRIPTION

The RNA polymerase complex was purified from influenza A/PR/8/34 as described in Section 6, supra. In vitro transcriptions of cold IVACAT1 or HgaNS RNA template were carried out using the conditions which have been described in Section 6, supra. Radiolabeled transcripts were analysed on 4% acrylamide gels.

7.1.5. RNP-TRANSFECTION OF MDCK AND 293 CELLS

35 mm dishes containing approximately 10^6 cells were treated with 1 ml of a solution of 300 µg/ml DEAE-dextrin, 0.5% DMSO in PBS/gelatine (0.1 mg/ml gelatine) for 30 minutes at room temperature. After removal of this solution, 200 µg of µl PBS/gelatine containing 1 µg IVACAT1 RNA (1-2 µl), 20 µl of the purified polymerase preparation and 4 µl of Rnasin was added to the cells and incubated for 1 hour at 37°C. This was followed by the addition of gradient purified influenza A/WSN/33 virus (moi 2-10). After incubation for one hour at 37° C, 2.5 ml of either DMEM + 10% FCS media (293 cells) or MEM media (MDCK cells) was added. In some experiments MDCK cells were first infected and subsequently RNP-transfected. Harvesting of cells was done in NET buffer or in media, using a rubber policemen (MDCK cells), or by gentle suspension (293 cells). Cells were spun down and the pellets were resuspended in 100 µl of 0.25 M Tris buffer, pH

7.5. The samples were subsequently freeze-thawed three-times and the cell debris was pelleted. The supernatant was used for CAT assays.

7.1.6. PASSAGING OF VIRUS FROM RNP-TRANSFECTED CELLS

MDCK cells were infected with helper virus and RNP-transfected 2 hours later as described above. After 1 hour cells and media were collected and cells were spun down. 100 µl of the supernatant media, containing virus, was added to 35 mm dishes with MDCK cells. After 12 hours these cells and media were collected and assayed for CAT activity. Virus contained in this supernatant media was used for subsequent rounds of infection of MDCK cells in 35 mm dishes.

7.1.7. CAT ASSAYS

CAT assays were done according to standard procedures, adapted from Gorman et al., 1982, Mol. Cell. Biol. 2: 1044-1051. The assays contained 10 µl of ^{14}C chloramphenicol (0.5 µCi; 8.3 nM; NEN), 20 µl of 40 mM acetyl CoA (Boehringer) and 50 µl of cell extracts in 0.25 M Tris buffer (pH 7.5). Incubation times were 16-18 hours.

7.2. RESULTS

rRNA templates were prepared from HgaI digested, end filled linearized pCATcNS using the bacteriophage T7 RNA polymerase as described in Section 6. The rRNA templates were combined with the viral RNA polymerase complex prepared as described in Section 6.1.1., and the resulting rRNPs were used to transfect MDCK and 293 cells lines which were superinfected with influenza A/WSN33. In each cell line transfected with the rRNPs, high levels of expression of CAT was obtained 6 hours post-infection. In addition, virus stocks obtained 24 hours post-infection synthesized high levels of CAT enzyme after subsequent passage in MDCK cells. The CAT-RNP was packaged into virus particles.

7.2.1. SYNTHESIS OF IVACAT1 TEMPLATE RNA

In order to study the transcription and replication signals of influenza A virus RNAs in vivo, we constructed plasmid pIVACAT1 (FIG. II) which directs the synthesis of an NS RNA-like transcript. This RNA shares the 22 5′ terminal and the 26′ 3′ terminal nucleotides with the NS RNA of influenza A/PR/8/34 virus and contain -- instead of the coding sequences for the NS1 and NS2 proteins -- those for a full-length CAT protein. For cloning purposes it also contains eight additional nucleotides including a BglII site between the stop codon of the CAT gene and the stretch of U's in the 5′ noncoding region. The T7 promoter adjacent to the 5′ noncoding sequences and the HgaI site downstream of the 3′ end allow for the exact tailoring of the 5′ and 3′ ends. Run-off transcription using T7 polymerase generates a 716 nt long RNA: Fig. 12, lanes 2-4 show that this RNA is of discrete length and shorter than the 890 nt long marker NS RNA, which was synthesized by T7 transcription of pHgaNS (lane 1).

7.2.2. THE IVACAT1 RNA IS TRANSCRIBED IN VITRO BY THE INFLUENZA A VIRUS RNA POLYMERASE

In the examples described in Section 6, it was demonstrated that synthetic RNAs containing at the 3′ end the 15 3′ terminal nucleotides of influenza virus RNA segment 8 can be transcribed in vitro using purified influenza A virus RNA polymerase. We tested whether unlabeled IVACAT1 RNA could be transcribed in a similar way. FIG. 12 lane 5 shows that the in vitro transcription reaction generated an RNA of discrete length and similar size to the product of the T7 transcription reaction suggesting synthesis of a full length product.

7.2.3. RNP-TRANSFECTION AND CAT ACTIVITY

Since the recombinant CAT RNA could be transcribed in vitro, a system was designed to test whether this RNA can be recognized and replicated in vivo (FIG. 13). Recombinant RNA was mixed with the purified polymerase to allow formation of viral RNP-like particles. To facilitate the association, the RNA/polymerase mixture was incubated in transcription buffer without nucleotides for 30 minutes at 30°C prior to RNP-transfection. In some experiments, this preincubation step was omitted. RNP-transfections were either preceeded or followed by infection with influenza A/WSN/33 virus, since the production of viral polymerase protein was expected to be necessary for efficient amplification of the gene. The cells used were either MDCK cells, which are readily susceptible to influenza A/WSN/33 virus infection, or human 293 cells, which support infection at a slower rate.

In order to determine whether the minus sense IVACAT1 RNA could be amplified and transcribed in vivo, an experiment was performed in 293 cells. Cells were transfected with RNP, virus infected one hour later and harvested at various times post-infection. FIG. 14A shows that at early times post infection only background levels of CAT activity were detected (lanes 5,7 and 9). However, significant levels of CAT activity appeared seven hours after virus infection (lane 11). A similar level of CAT activity was detected two hours later (lane 13). There were background levels of CAT activity in the mock transfected cells at any time point (lanes 6, 8, 10, 12 and 14), and in control cells not infected with A/WSN/33 virus (lanes 1-4).

Preincubation of RNA and polymerase complex was not necessary for successful RNP-transfection. As can be seen in Fig. 14B, lanes 2 and 3, preincubation might actually cause a decrease in CAT activity, presumably due to RNA degradation during preincubation. In another control experiment, infection by helper virus of RNP-transfected cells was

omitted (FIG. 14B, lanes 4 and 5). Since these lanes show no CAT activity we conclude that the IVACAT1 RNA is amplified specifically by the protein machinery supplied by the helper virus. In an additional control experiment, naked RNA was transfected into cells which were subsequently helper-infected or mock-infected. Again, no CAT activity was detected in these samples (FIG. 14B, lanes 6-9). Finally, virus-infected cells which were not transfected with recombinant CAT-RNP also did not exhibit endogneous acetylation activity (FIG. 14B, lane 10). It thus appears that addition of the purified polymerase to the recombinant RNA as well as infection of cells by helper virus is important for successful expression of the CAT enzyme.

Experiments were also performed using MDCK cells, the usual tissue culture host cell for influenza virus (FIG. 14C). When the reconstituted recombinant CAT-RNP complex was transfected 1 hour before virus infection, little CAT activity was observed at 7 hours post virus infection (FIG. 14C, lane 1). When RNP-transfection was accomplished 2 hours after virus infection, expression of CAT was greatly enhanced at 7 hours post-virus infection (FIG. 14C, lane 3). Therefore, MDCK cells are also viable host cells for these experiments.

7.2.4. THE CAT-RNP IS PACAKAGED INTO VIRUS PARTICLES

Since the recombinant CAT RNA can be replicated in vivo via helper virus functions, we examined whether virus produced in RNP-transfected and helper virus infected cells contained the CAT gene. MDCK cells were used in the experiment because they yield higher titers of infectious virus than 293 cells. MDCK cells were infected with A/WSN/33 virus, RNP-transfected 2 hours later and allowed to incubate overnight. At 14 hours post infection, media was harvested and cells were pelleted. Virus supernatant was then used to infect new MDCK cell monolayers. The inoculum was removed

after 1 hour and cells were harvested at 12 hours post infection and assayed for CAT activity. FIG. 15 reveals that the virus preparation induces a level of CAT activity (lanes 2 and 3) which is significantly above control (lane 1). In this case, the addition of helper virus to the inoculum did not increase CAT activity (lane 4). Further passaging of supernatant virus on fresh MDCK cells did not result in measurable induction of CAT activity. This is not surprising as there is no selective pressure for retaining the CAT gene in these viral preparations. We excluded the possibility that we were transferring the original RNA/polymerase complex by pretreating the inocula with RNase. This treatment destroys viral RNPs of influenza virus (Pons et al. 1969 Virology 39: 250-259; Scholtissek and Becht, 1971 J. Gen. Virol. 10: 11-16).

8. RESCUE OF INFECTIOUS INFLUENZA VIRUSES USING RNA DERIVED FROM SPECIFIC RECOMBINANT DNAs

The experiments described in the subsections below demonstrate the rescue of infectious influenza viruses using RNA which is derived from specific recombinant DNAs. RNAs corresponding to the neuraminidase (NA) gene of influenza A/WSN/33 virus (WSN virus) were transcribed in vitro from appropriate plasmid DNAs and -- following the addition of purified influenza virus polymerase complex (as described in Section 6.1.1. supra) -- were transfected into MDBK cells as described in Section 7, supra. Superinfection with helper virus, lacking the WSN NA gene, resulted in the release of viruses containing the WSN NA gene. Thus, this technology allows the engineering of infectious influenza viruses using cDNA clones and site-specific mutagenesis of their genomes. Furthermore, this technology may allow for the construction

of infectious chimeric influenza viruses which can be used as efficient vectors for gene expression in tissue culture, animals or man.

The experiments described in Sections 6 and 7 supra, demonstrate that the 15 3′ terminal nucleotides of negative strand influenza virus RNAs are sufficient to allow transcription in vitro using purified influenza virus polymerase proteins. In addition, the studies using the reporter gene chloramphenicol acetyltransferase (CAT) show that the 22 5′ terminal and the 26 3′ terminal nucleotides of the viral RNAs contain all the signals necessary for transcription, replication and packaging of influenza virus RNAs. As an extension of these results, a plasmid, pT3NAv, was constructed which contained the complete NA gene of influenza A/WSN/33 virus downstream of a truncated T3 promoter (FIG. 16). Therefore, runoff transcription of this plasmid, cut at the Ksp632I site, yields an RNA which is identical to the true genomic NA gene of the WSN virus (Fig. 17, lane 3). This RNA was then incubated with purified polymerase (purified as described in Section 6.1.1) and used in a ribonucleoprotein (RNP) transfection experiment to allow the rescue of infectious virus using helper virus which did not contain the WSN virus NA. The choice of WSN-HK helper virus was based on the need for a strong selection system by which to isolate a rescued virus. Previously, it was shown that the WSN-HK virus can only form plaques in MDBK cells when protease is added to the medium. This is in marked contrast to WSN virus (isogenic to WSN-HK virus except for the neuraminidase gene), which in the absence of protease readily replicates in MDBK cells and forms large, easily visible plaques (Schulman et al., 1977, J. Virol. 24:170-176).

8.1. MATERIALS AND METHODS

8.1.1. VIRUSES AND CELLS

Influenza A/WSN/33 virus and A/WSN-HK virus were grown in Madin-Darby canine kidney (MDCK) cells and embryonated eggs, respectively (Sugiura et al., 1972, J. Virol. 10:639-647; Schulman et al., 1977, J. Virol. 24:170-176. Influenza A/PR/8/34 virus was also grown in embryonated eggs. Madin-Darby bovine kidney (MDBK) cells were used for the transfection experiments and for selection of rescued virus (Sugiura et al., 1972, J. Virol. 10:639-647).

8.1.2. CONSTRUCTION OF PLASMIDS

The pT3NAv, pT3NAv mut 1 and pT3NAv mut 2 plasmids were constructed by PCR-directed mutagenesis using a cloned copy of the WSN NA gene, which was obtained following standard procedures (Buonagurio et al., 1986, Science 232:980-982). To construct pT3NAv, the following primers were used: 5′-CGGAATTCTCTTCGAGCGAAAGCAGGAGTT-3′ and 5′-CCAAGCTTATTAACCCTCACTAAAAGTAGAAACAAGGAGTTT-3′. After 35 cycles in a thermal cycler (Coy Lab products, MI), the PCR product was digested with EcoRI and HindIII and cloned into pUC19. Plasmid pT3NAv mut 1 was constructed in a similar fashion except that the sequence of the primer was altered (FIG. 16). Plasmid pT3NAv mut 2 was constructed by cassette mutagenesis through the digestion of pT3NAv with PstI and NcoI and religation in the presence of the synthetic oligonucleotides - 5′-CATGGGTGAGTTTCGACCAAAATCTAGATTAT-AAAATAGGATACATATGCA-3′ and 5′-AATGTATCCTATTTTATAATC-TAGATTTTGGTCGAAACTCACC-3′. Oligonucleotides were synthesized on an applied Biosystems DNA synthesizer. The final clones pT3NAv, pT3NAv mut 1 and pT3NAv mut 2 were grown up and the DNAs were partially sequenced starting from the flanking pUC19 sequences and reaching into the coding sequences of the NA gene. The mutations in pT3NAv mut 2 were also confirmed by sequencing.

8.1.3. PURIFICATION OF INFLUENZA A VIRUS POLYMERASE AND RNP TRANSFECTION IN MDBK CELLS

The RNA polymerase complex was purified from influenza A/PR/8/34 virus as described in Section 6.1.1, supra, and was then used for RNP transfection in MDBK cells using the protocol described in Section 7, supra, except that WSN-HK virus was used as helper virus at an moi of 1. RNAs used for RNP transfection were obtained by phenol extraction of purified virus or by transcription (using T3 polymerase) of pT3NAv, pT3NAv mut 1 and pT3NAv mut 2. All plasmids were digested with Ksp632I, end-filled by Klenow enzyme (BRL) and then transcribed in a runoff reaction as described in Section 7, supra.

8.2. RESULTS

8.2.1. RESCUE OF INFECTIOUS INFLUENZA VIRUS IN MDBK CELLS USING RNA DERIVED FROM RECOMBINANT PLASMID DNA

A plasmid, pT3NAv, was constructed to contain the complete NA gene of influenza WSN virus downstream of a truncated T3 promoter (FIG. 16). Runoff transcription of the plasmid, cut at the Ksp632I site, yields an RNA which is identical in length to the true genomic NA gene of the WSN virus (FIG. 17, lane 3). This RNA was then incubated with purified polymerase and used in a ribonucleoprotein (RNP) transfection experiment to allow the rescue of infectious virus using helper virus. The choice of WSN-HK virus as helper virus was based on the need for a strong selection system by which to isolate a rescued virus. Previously, it was shown that the WSN-HN virus can only form plaques in MDBK cells when protease is added to the medium (Schulman et al., 1977, J. Virol. 24:170-176). This is in marked contrast to WSN virus (isogenic to WSN-HK helper virus except for the neuraminidase gene), which is the absence of protease readily replicates in MDBK cells and forms large, easily visible

plaques (Sugiura et al., 1972, J. Virol. 10:639-647). MDBK cells were first infected with the WSN-HK helper virus and RNP-transfected one hour after virus infection. Following overnight incubation in the presence of 20μg/ml plasminogen, supernatant from these cells was then amplified and plaqued in MDBK cells in the absence of protease in the medium. The appearance of plaques in MDBK cells (Schulman et al., 197, J. Virol. 10:639-647) indicated the presence of virus which contained the WSN virus NA gene, since supernatant from control experiments of cells infected only with the WSN-HK virus did not produce plaques. In a typical experiment involving the use of a 35 mm dish for the RNP-transfection, 2.5×10^2 plaques were observed.

In another control experiment, synthetic NA RNA was used which was derived from plasmid pT3NAv mut 1 (FIG. 16). This RNA differs from the wild type NA RNA derived from pT3NAv by a single nucleotide deletion in the nontranslated region of the 5′ end (FIG. 16). RNP-transfection of MDBK cells with this RNA and superinfection with WSN-HK virus did not result in the formation of rescued virus. This negative result is readily explained since we have shown in Sections 6 and 7, supra, that the essential sequences for the recognition of viral RNA by viral polymerases as well as the packaging signals are located within the 3′ and 5′ terminal sequences of the viral RNAs. However, we cannot exclude the possibility that rescue of virus using this mutated RNA does occur, albeit at an undetected frequency.

8.2.2. RNA ANALYSIS OF RESCUED VIRUS

Virus obtained in the rescue experiment was plaque purified, amplified in MDBK cells and RNA was extracted from this preparation. The RNA was then analyzed by electrophoresis on a polyacrylamide gel. FIG. 17 shows the RNA of the helper virus WSN-HK (lane 1) and the synthetic NA RNA (lane 3), which was transcribed by T3 polymerase from

plasmid pT3NAv. The migration pattern of the RNAs of the rescued virus (lane 2) is identical to that of control WSN virus (lane 4). Also, the NA RNAs in lanes 2 and 4 migrate at the same position as the NA RNA derived from cDNA (lane 3) and faster than the HK virus NA band in the helper WSN-HK virus (lane 1). These experiments support the conclusion that as a result of the RNP-transfection, infectious virus was formed containing WSN virus NA RNA derived from cDNA.

8.2.3. RESCUE OF INFECTIOUS INFLUENZA VIRUS USING VIRION RNA

In another transfection experiment, RNA extracted from purified WSN virus was employed. When this naked RNA is transfected together with the polymerase proteins into helper virus infected cells, rescue of WSN virus capable of replicating in MDBK cells is observed. RNA isolated from an amplified plaque in this experiment is analyzed in lane 5 of FIG. 17 and shows a pattern indistinguishable from that of the control of WSN virus in lane 4.

8.2.4. INTRODUCTION OF SITE-SPECIFIC MUTATIONS INTO THE VIRAL GENOME

The experiments described so far involved the rescue of influenza WSN virus. Since the synthetic RNA used in these experiments is identical to the authentic WSN NA gene, the unlikely possibility of contamination by wild type WSN virus could not be rigorously ruled out. Therefore, we introduced five silent point mutations into the coding region of the NA gene in plasmid pT3NAv. These mutations were introduced by cassette mutagenesis through replacement of the short NcoI/PstI fragment present in the NA gene. The five mutations in the cDNA included a C to T change at position 901 and a C to A change at position 925, creating a new XbaI site and destroying the original PstI site, respectively. In addition, the entire serine codon at position 887-889 of the

cDNA clone was replaced with an alternate serine triplet (FIG. 17). RMP-transfection of this mutagenized RNA (pT3NAv mut 2) and helper virus infection of MDBK cells again resulted in the rescue of a WSN-like virus which grew in MDBK cells in the absence of added protease. When the RNA of this virus was examined by sequence analysis, all five point mutations present in the plasmid DNA (FIG. 16) were observed in the viral RNA (FIG. 18). Since it is extremely unlikely that these mutations evolved in the wild type influenza WSN virus, we conclude that successful rescue of infectious influenza virus containing five site-specific mutations was achieved via RNP-transfection of engineered RNA.

9. EXAMPLE: SYNETHETIC REPLICATION SYSTEM

In the experiments described below, a cDNA clone which can produce an influenza virus-like vRNA molecule coding for a reporter gene was used. This resultant RNA is an NS-like vRNA which contains the antisense of the coding region of the chloramphenicol acetyltransferase gene (CAT) in place of the antisense coding regions for the nonstructural proteins, NS1 and NS2 (Lutjyes et al., 1989, Cell, 59: 1107-1113). This recombinant RNA (IVACAT-1) was incubated with purified influenza virus RNP proteins and used in an attempt to develop a non-influenza virus dependent replication system. Mouse fibroblast C127 cells were infected with mixtures of recombinant vaccinia viruses (Smith et al., 1987, Virology, 160: 336-345) and transfected one hour later with the IVACAT-1 RNP. Mixtures of vectors expressing the three polymerases (PB2, PB1 and PA) and the nucleoprotein were used. Replication and transcription of the synthetic RNP was assayed by analyzing cells for CAT activity after overnight incubation. Figure 19 examines the CAT activity present in cells initially infected with many of the possible mixtures of the 4 recombinant vaccinia viruses. Figure 19, lane 4 is a positive control in which the influenza A/WSN/33 virus was

used in lieu of the recombinant vaccinia viruses. CAT activity is present in this sample as well as in cells infected with all four vaccinia vectors (Figure 19, lanes 8 and 10). Cells expressing any of the subsets of these four proteins did not produce detectable CAT protein (Figure 19, lanes 5-7, 9, 11, unpublished). In addition, transfected RNA not incubated with the purified polymerase was also negative for CAT expression (Lutjyes, et al. 1989). Thus, the presence of the PB2, PB1, PA and NP proteins are all that is necessary and sufficient for RNP expression and replication in this system. The levels of CAT activity obtained in vaccinia vector-infected cells are reproducibly higher than in cells infected with influenza as helper virus. The most probable explanation for this is that in influenza virus-infected cells, the CAT-RNP competes with the endogenous viral RNP's for active polymerase whereas in the vaccinia driven system that CAT-RNP is the only viral-like molecule present.

A number of other cell lines were then tested as hosts for this vaccinia virus driven system. Figure 20A shows the results using MDBK, Hela, 293 and L cells. In each case, no CAT activity was observed when cells were infected with vectors that express only the 3 polymerase proteins but significant CAT activity was obtained if the additional vaccinia-vector inducing NP expression was also added.

Previously, a cell line (designated 3PNP-4) was constructed which constitutively expresses low levels of the PB2, PB1 and PA proteins and high levels of the NP protein. These cells can complement the growth of ts mutants mapping either to the PB2 or NP gene segments (Krystal et al., 1986; Li et al., 1989). Since replication through recombinant vaccinia virus vectors is dependent only on these proteins, it was conceivable that this cell line may be able to amplify and express the synthetic CAT-RNP in the absence of any virus infection. However, when this experiment was attempted, no

detectable CAT activity was obtained (data not shown). In order to investigate the reasons why this cell line did not support replication, mixtures of recombinant vaccinia viruses were used to infect 3PNP-4 cells. As expected, the addition of the four polymerase proteins supported the expression of CAT (Fig. 20B, lane 2). Figure 20B, lane 3 shows that the minimum mixture of vectors needed to induce CAT activity in 3PNP-4 cells are those expressing only the PB1 and PA proteins. Therefore, the steady state levels of PB2 and NP proteins in 3PNP-4 cells are sufficient but the levels of PB1 and PA are below threshold for CAT expression in the absence of helper virus. This correlates with the complementation phenotype exhibited by these cells, since only the growth of PB2 and NP mutants and not PB1 and PA mutants can be recovered at non-permissive temperature (Desselberger et al., 1980).

Since the synthetic IVACAT-1 RNA is of negative polarity, CAT can only be synthesized via transcription off the RNP molecule. Theoretically, detectable levels of CAT can be produced either through transcription off the transfected input RNP (equivalent to primary transcription) or first through amplification of RNP and subsequent transcription (necessitating RNP replication) or a combination of both. However, previous work using influenza virus infection to drive the expression of the CAT protein showed that detectable expression occurred only if the input CAT-RNP was replicated (Lutjyes et al., 1989). This was shown by the use of a second CAT-RNA, IVACAT-2, which contains 3 mutations within the 12 bases at the 5′ end of the viral RNA (Lutjyes et al., 1989). This 12 base region is conserved among all eight gene segments in all influenza A viruses (Desselberger, at al., 1980). This synthetic IVACAT-2 RNP is competent for transcription by the influenza virus polymerase but it is not replicated and when transfected into influenza virus-infected cells CAT activity

remained undetected (Lutyjes et al., 1989). Therefore, primary transcription off the input RNA does not produce detectable levels of protein in influenza virus infected cells. Accordingly, we used this mutant RNA to examine whether the vaccinia vector-expressed influenza proteins induces CAT activity solely through primary transcription of input RNP or can allow for amplification through replication and subsequent transcription. C127 cells were infected with the recombinant vaccinia viruses and then transfected with either IVACAT-1 and IVACAT-2 generated RNPs. Figure 20C shows that low levels of CAT activity can be detected in cells transfected with IVACAT-2 RNP (lane 2). When quantitated, 0.5-1% of the chloramphenical is converted to an acetylated form, compared to 0.2-0.4% in mock transfected lanes (not shown). However, much greater levels of activity are present in cells transfected with CAT-1 RNP (lane 1; routinely 15-50% conversion of chloramphenical), indicating that amplification is occurring in these cells. Therefore, this recombinant vaccinia virus-driven system is sequence-specific and the RNP's are undergoing replication.

In the experiments described, neither the NS1 nor NS2 proteins were required for RNP replication. Although their function is not known it has been speculated that these proteins may play a major role in replication because both proteins are synthesized in large amounts and are present in the nucleus (Krug et al., 1989; Young et al., 1983, Greenspan et al., 1985; Lamb et al., 1984). Based on the data presented, these proteins are not absolutely required for genome replication. It may be speculated that these proteins may actually have ancillary roles with regard to the replication of RNP, such as interaction with host factors, regulation of the expression of viral genes or some function involved with packaging of the RNP into infectious virions. However, it can not be ruled out that a function of these NS proteins may be complemented by a vaccinia virus protein,

although upon inspection, no obvious similarities were found between either the NS1 or NS2 proteins and known vaccinia virus proteins. The contrasting properties of these two viruses also argues against a complimenting vaccinia virus protein, as vaccinia is a large double-stranded DNA virus replicating exclusively in the cytoplasm while influenza virus is a negative sense RNA virus replicating exclusively in the nucleus. In addition, the replication of the synthetic RNPs occurred even in the presence of cytosine arabinoside (ara-C, data now shown), an inhibitor of late gene expression in vaccinia virus (Oda et al., 1967; Kaverin et al., 1975; Cooper et al., 1979).

This recombinant vaccinia vector dependent scheme possesses a number of advantages over the use of influenza virus infection to drive the replication of synthetic RNA. For one, since the expression of the viral proteins is completely artificial it will allow for a precise dissection of the processes involved in replication. Replication first involves the synthesis of positive sense template from the negative sense genomic RNA. This positive sense cRNA is then copied in order to amplify genomic sense RNP, which is then used for protein expression and packaging (Krug et al., 1989). The system described herein demonstrate that only the influenza viral PB2, PB1, PA and NP proteins are required for the detection of expressed protein and for replication of RNP. Another advantage of this vaccinia vector driven replication scheme is that since the influenza polymerase proteins are expressed from cDNA integrated into the vaccinia virus, the mutagenesis of the polymerase proteins becomes a feasible and powerful method to further analyze structure-function relationships of the viral polymerase proteins. Also, we are currently attempting to rescue infectious influenza virus through the transfection of mixtures of reconstituted viral RNPs. This technique, if successful, should allow for the easy construction of defined recombinant

viruses through the addition of defined RNPs, either naturally or synthetically derived. This technology should also be applicable for the analysis and dissection of the replication apparatus of other negative strand viruses.

10. DEPOSIT OF MICROORGANISMS

An E. coli cell line containing the plasmid pIVACAT is being deposited with the Agricultural Research Culture Collection (NRRL), Peoria, IL; and has the following accession number

Strain	Plasmid	Accession Number
E. coli (DH5a)	pIVACAT	NRRL B-18540

The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and any constructs, viruses or enzymes which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

International Application No: PCT/ /

MICROORGANISMS

Optional Sheet in connection with the microorganism referred to on page 85, line 12 of the description [1]

A. IDENTIFICATION OF DEPOSIT [2]

Further deposits are identified on an additional sheet ☐ [2]

Name of depositary institution [3]

Agricultural Research Culture Collection

Address of depositary institution (including postal code and country) [3]

1815 N. University Street
Peoria, Illinois 61604

Date of deposit [4]	Accession Number [5]
December 6, 1989	NRRL B-18540

B. ADDITIONAL INDICATIONS [1] (leave blank if not applicable). This information is continued on a separate attached sheet ☐

C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE [1] (if the indications are not for all designated States)

D. SEPARATE FURNISHING OF INDICATIONS [6] (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later [1] (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☒ This sheet was received with the international application when filed (to be checked by the receiving Office)

Doris L. Brock
(Authorized Officer)

☐ The date of receipt (from the applicant) by the International Bureau is

was

(Authorized Officer)

Form PCT/RO/134 (January 1981)

WHAT IS CLAIMED IS:

1. A recombinant, negative-strand RNA template comprising an RNA-directed RNA polymerase binding site ligated to a heterologous RNA sequence representing the opposite polarity of its message, so that the polymerase binding site is located upstream of the start of the corresponding transcribed message.

2. The recombinant, negative strand RNA template of Claim 1 in which the polymerase binding site comprises the 3′-noncoding viral sense flanking sequence of an influenza genome segment.

3. The recombinant, negative strand RNA template of Claim 1 in which the polymerase binding site comprises the terminal 15 nucleotides of the 3′-terminus of an influenza genomic segment.

4. The recombinant, negative-strand RNA template of Claim 1 in which the 3′-noncoding viral sense flanking sequence of influenza comprises the following sequence:

5′-CACCCUGCUUUUGCU-3′

5. The recombinant, negative-strand RNA template of Claim 1 in which the 3′-noncoding viral sense flanking sequence of influenza comprises the following sequence:

5′-CACCCUGCUUCUGCU-3′

6. The recombinant, negative-strand RNA template of Claim 1 in which the 3′-noncoding viral sense flanking sequence of influenza comprises the following sequence:

5′-CACCCUGUUUUUGCU-3′

7. The recombinant, negative-strand RNA template of Claim 1 in which the 3′-noncoding viral sense flanking sequence of influenza comprises the following sequence:

5′-CACCCUUGCUUUUGCU-3′

8. A recombinant, negative-strand RNA template comprising a heterologous RNA sequence representing the opposite polarity of its message ligated to a 3′- noncoding viral sense flanking sequence of influenza containing the viral polymerase binding site, and to a 5′-noncoding viral sense flanking sequence of influenza so that the viral polymerase binding site is located upstream of the start of the corresponding transcribed message.

9. The recombinant, negative-strand RNA template of Claim 8 in which the 5′-noncoding viral sense flanking sequence of influenza comprises the first 22 nucleotides of the 5′-terminus of an influenza genomic segment.

10. The recombinant, negative-strand RNA template of Claim 8 in which the 5′-noncoding viral sense flanking sequence of influenza comprises the following sequence:

5′-AGUAGAAACAAGGGUGUUUUUU-3′.

11. A recombinant RNP comprising the recombinant template of Claim 1 mixed with purified influenza viral polymerase.

12. The recombinant RNP of Claim 11 in which the influenza viral polymerase is obtained from RNPs fractionated by centrifugation on a CsCl gradient, in which the purified influenza viral polymerase is isolated from the region of the gradient correlating to 1.5 to 2.0 M CsCl.

13. A recombinanant RNP comprising the recombinant template of Claim 8 mixed with purified influenza viral polymerase.

14. The recombinant RNP of Claim 13 in which the influenza viral polymerase is obtained from RNPs fractionated by centrifugation on a CsCl gradient, in which the purified influenza viral polymerase is isolated from the region of the gradient correlating to 1.5 to 2.0 M CsCl.

15. A chimeric virus comprising influenza virus containing a heterologous RNA sequence representing the opposite polarity of its message ligated to an influenza viral polymerase binding site, so that the viral polymerase binding site is located upstream of the start of the corresponding transcribed message.

16. The chimeric virus of Claim 15 in which the heterologous RNA sequence is contained within segment 1 of influenza.

17. The chimeric virus of Claim 15 in which the heterologous RNA sequence is contained within segment 2 of influenza.

18. The chimeric virus of Claim 15 in which the heterologous RNA sequence is contained within segment 3 of influenza.

19. The chimeric virus of Claim 15 in which the heterologous RNA sequence is contained within segment 4 of influenza.

20. The chimeric virus of Claim 15 in which the heterologous RNA sequence is contained within segment 5 of influenza.

21. The chimeric virus of Claim 15 in which the heterologous RNA sequence is contained within segment 6 of influenza.

22. The chimeric virus of Claim 15 in which the heterologous RNA sequence is contained within segment 7 of influenza.

23. The chimeric virus of Claim 15 in which the heterologous RNA sequence is contained within segment 8 of influenza.

24. A chimeric virus comprising influenza virus containing in addition to its eight genomic segments an additional RNA segment containing a heterologous gene represented in the negative polarity flanked by the influenza viral polymerase binding site, so that the viral polymerase binding site is located upstream of the start of the corresponding transcribed message.

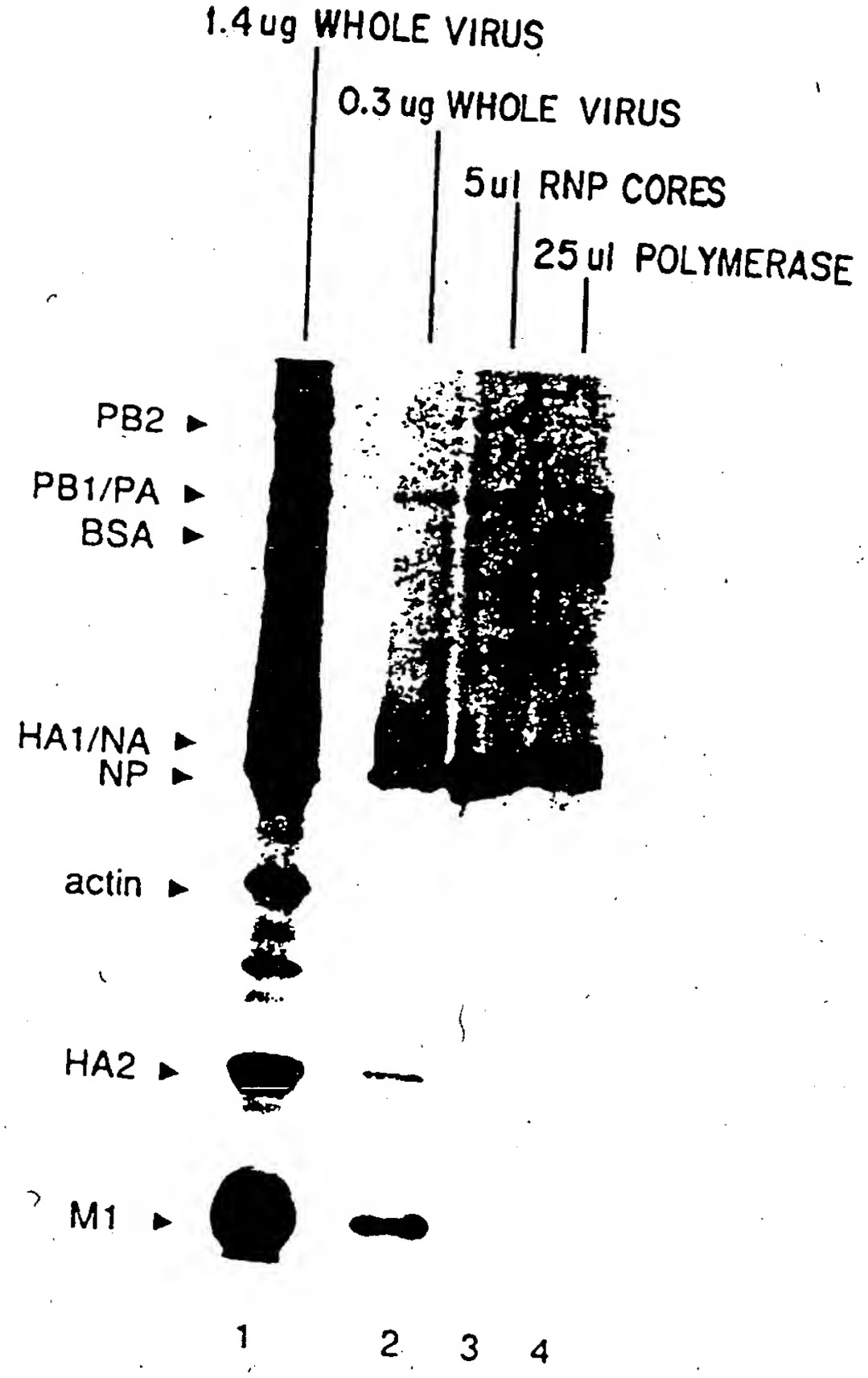

1.4 ug WHOLE VIRUS
0.3 ug WHOLE VIRUS
5 ul RNP CORES
25 ul POLYMERASE
PB2
PB1/PA
BSA
HA1/NA
NP
actin
HA2
M1
1
2
3
4

FIG. 1

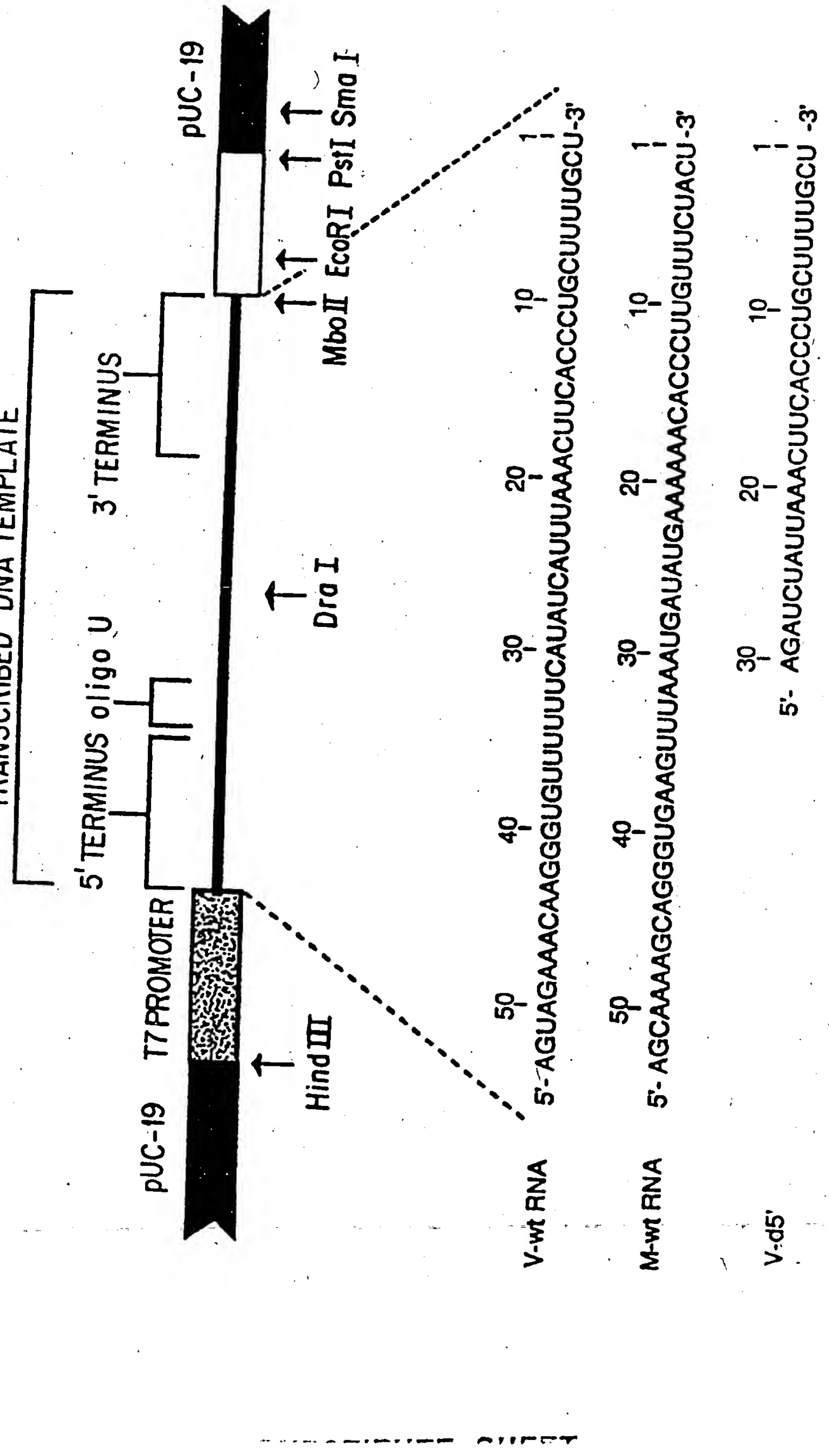

pUC-19
TRANSCRIBED DNA TEMPLATE
5' TERMINUS oligo U
3' TERMINUS
T7 PROMOTER
pUC-19
HindIII
DraI
MboII EcoRI PstI SmaI
V-wt RNA
M-wt RNA
V-d5'
5'- AGUAGAAACAAGGGUGUUUUUCAUAUCAUUUAAACUUCACCCUGCUUUUGCU-3'
5'- AGCAAAAGCAGGGUGAAGUUUAAAUGAUAUGAAAAAACACCCUUGUUUUCUACU-3'
5'- AGAUCUAUUAAACUUCACCCUGCUUUUGCU -3'
1
10
20
30
40
50

FIG. 2

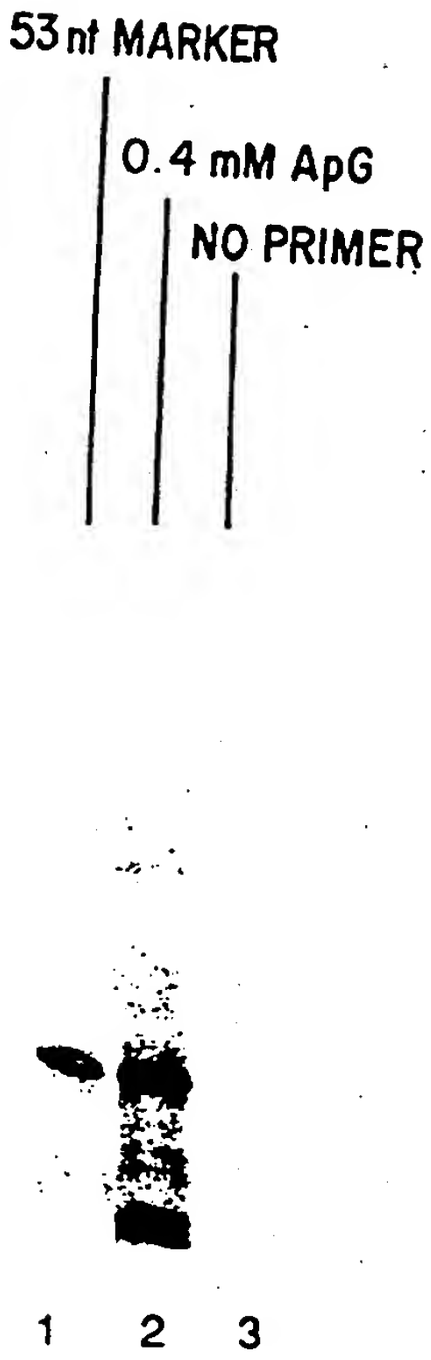


FIG. 3A

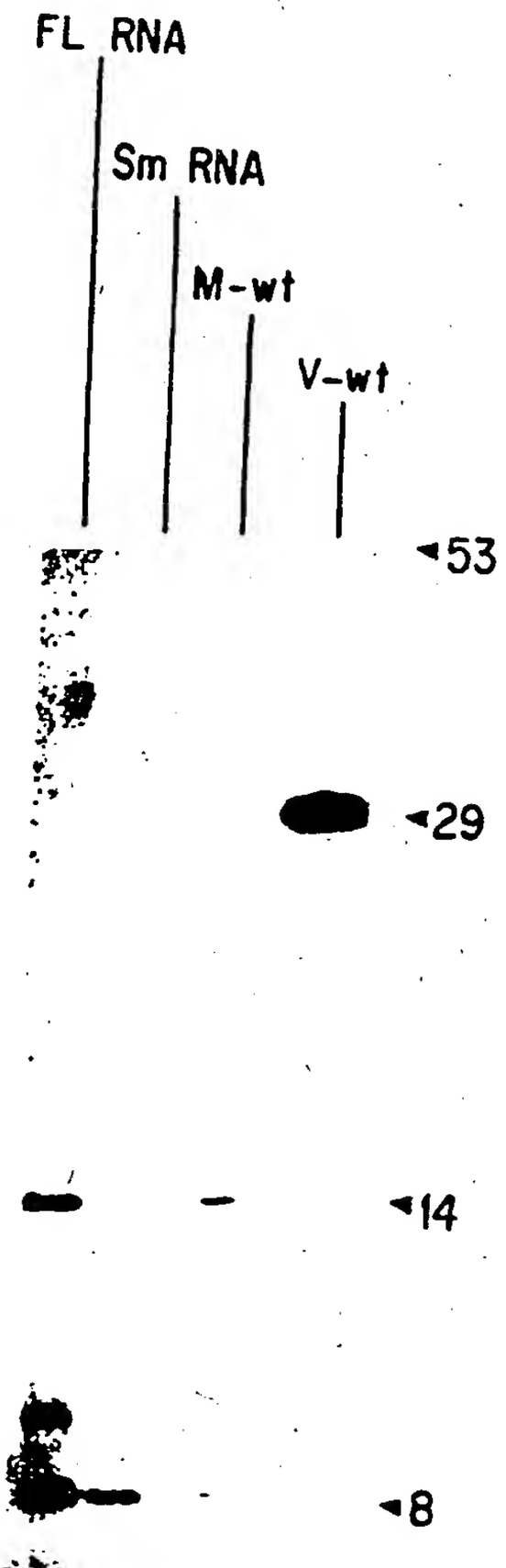


FIG. 3C

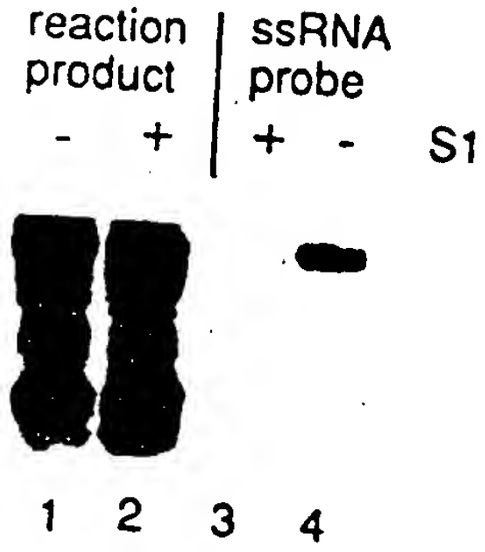


FIG. 3B

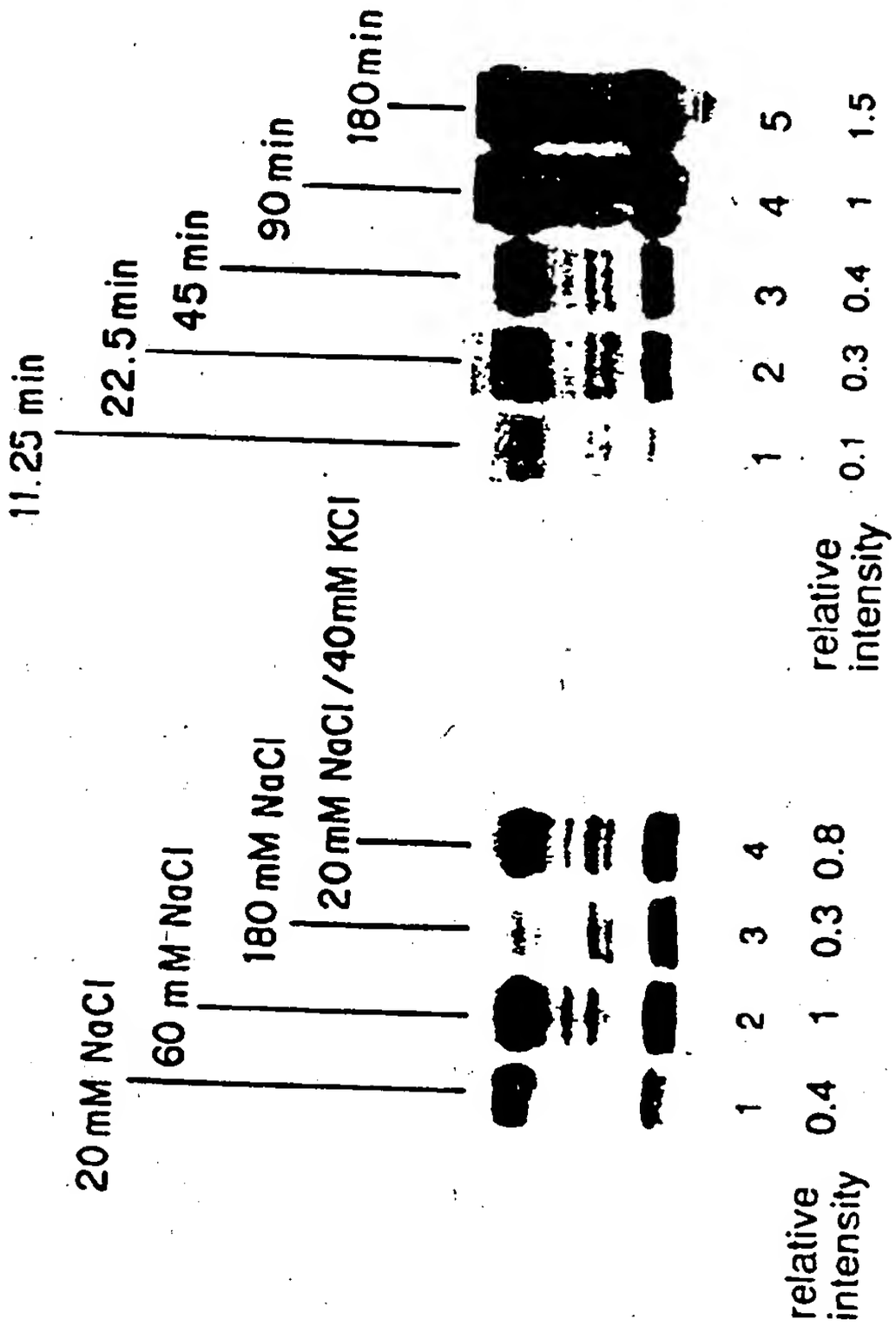


FIG. 4C

FIG. 4B

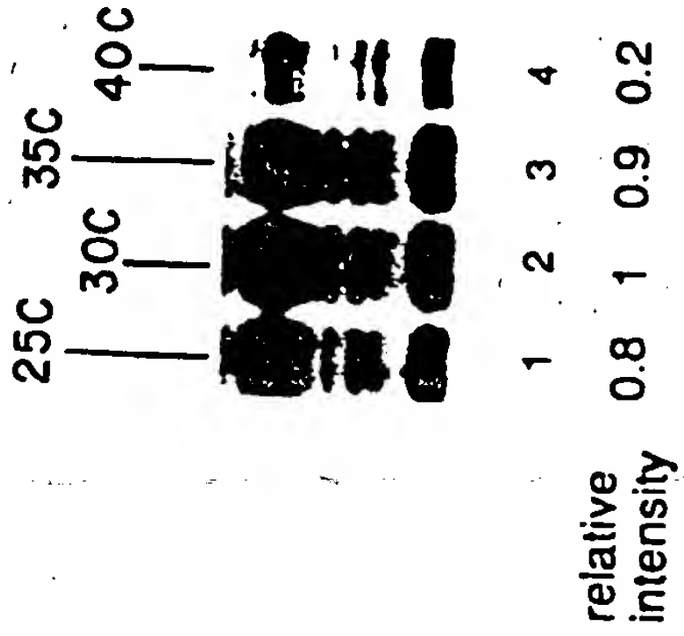


FIG. 4A


V-Pst
V-Sma
V-wt
V-wt DNA
IBI-31
1 2 3 4 5

FIG. 5A

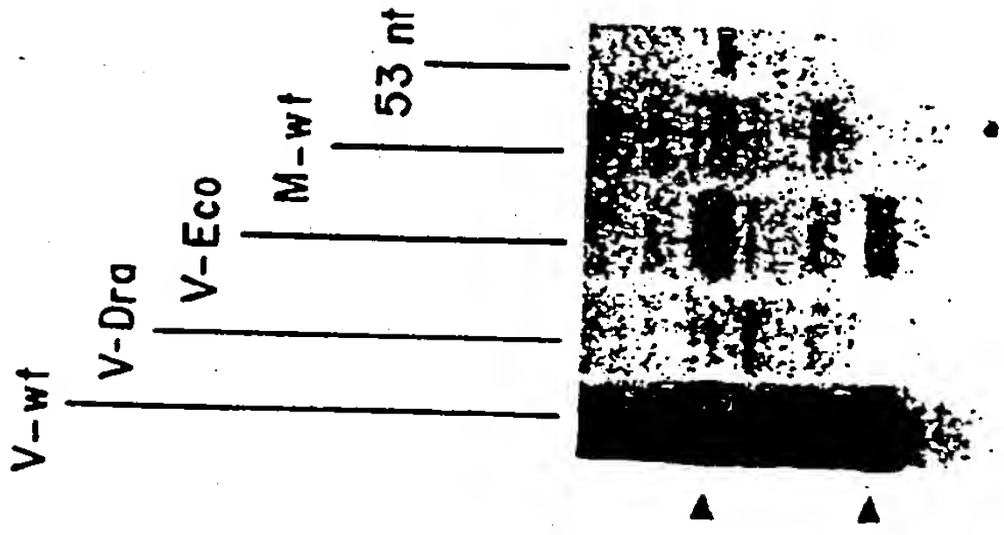
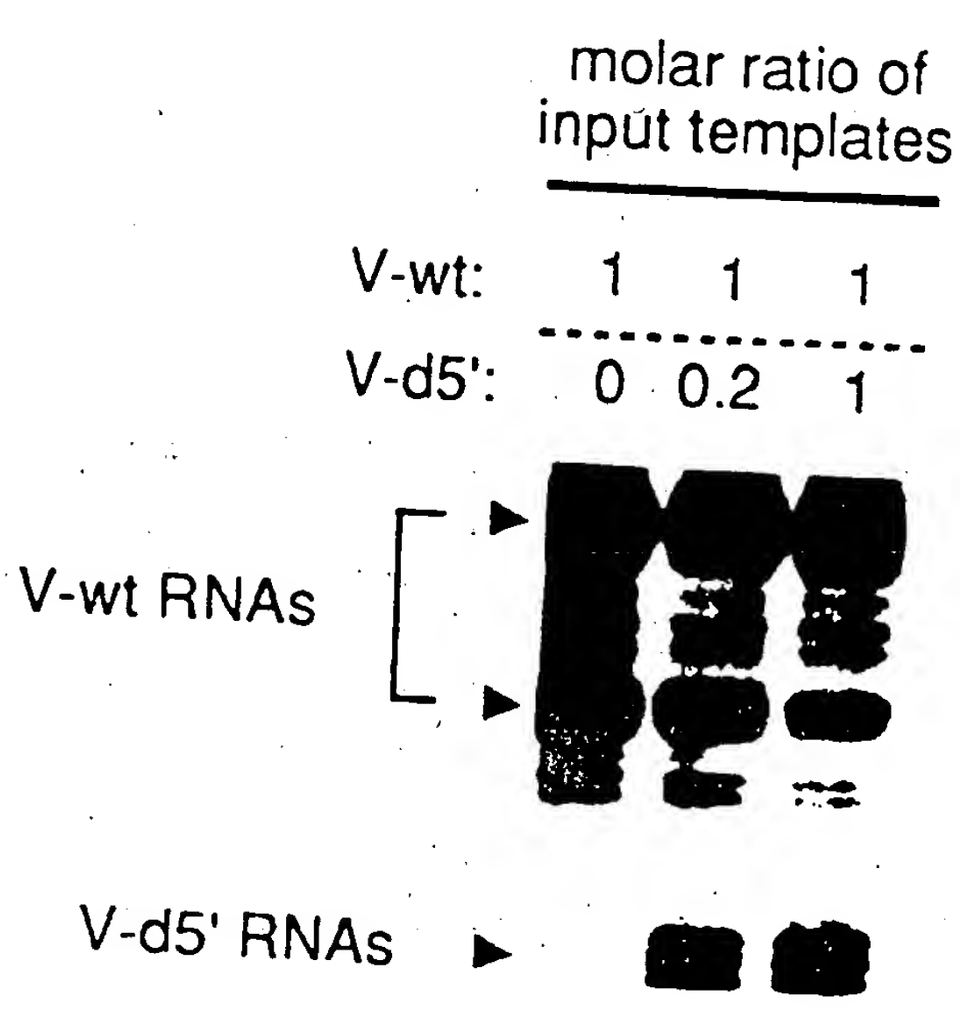

V-wt
V-Dra
V-Eco
M-wt
53 nt
ssRNA
1 2 3 4 5

FIG. 5B



corrected relative intensities	1	2	3
V-wt:	1	0.8	0.6
V-d5':	0	0.2	0.4

FIG. 6

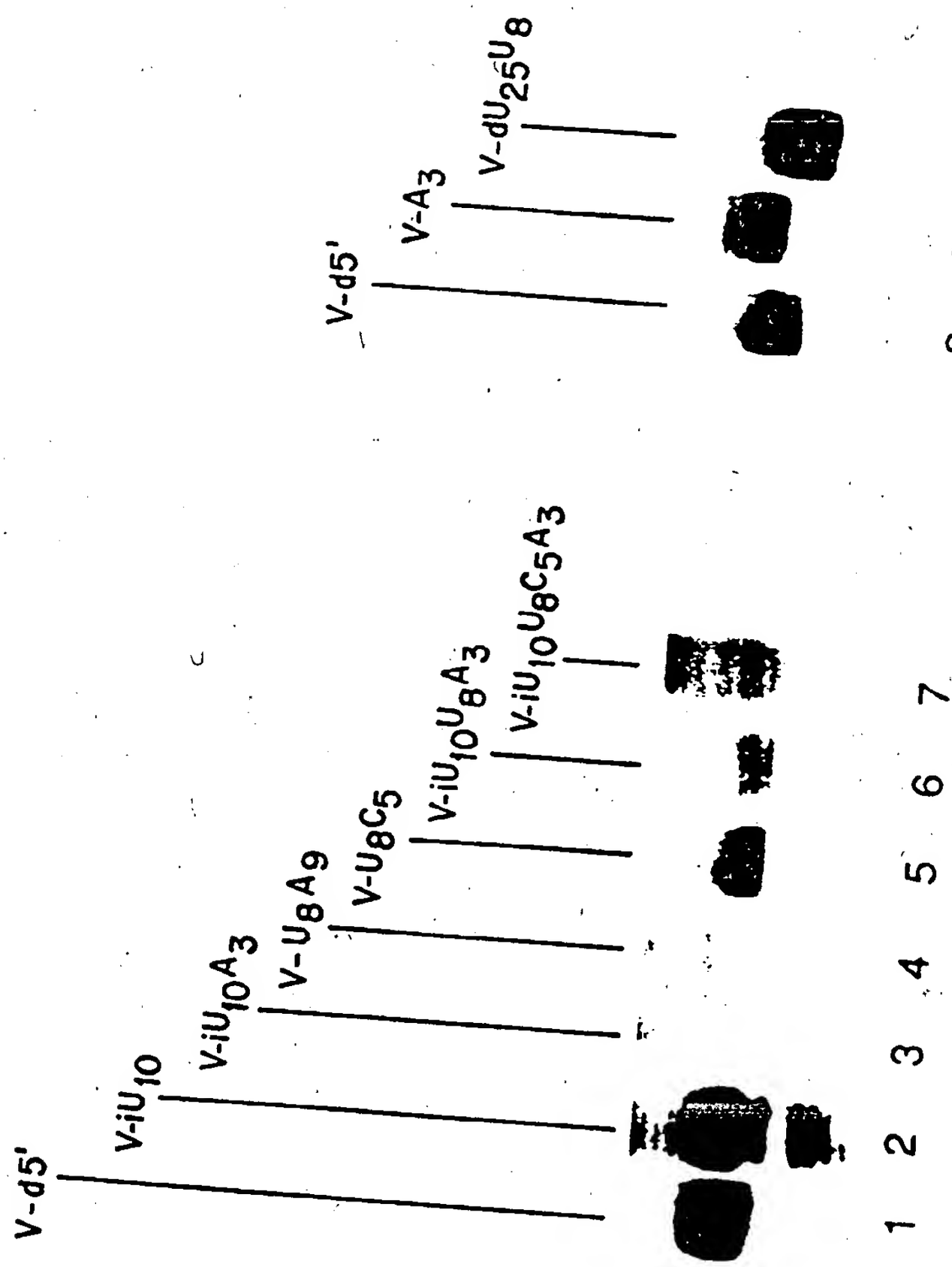

V-d5'
V-A3
V-dU2
V-iU10U8C5A3
V-iU10U8A3
V-U8C5A9
V-iU10
V-iU10A3
V-U
V-d5'
1 2 3 4
5 6 7
8 9 10

FIG. 7

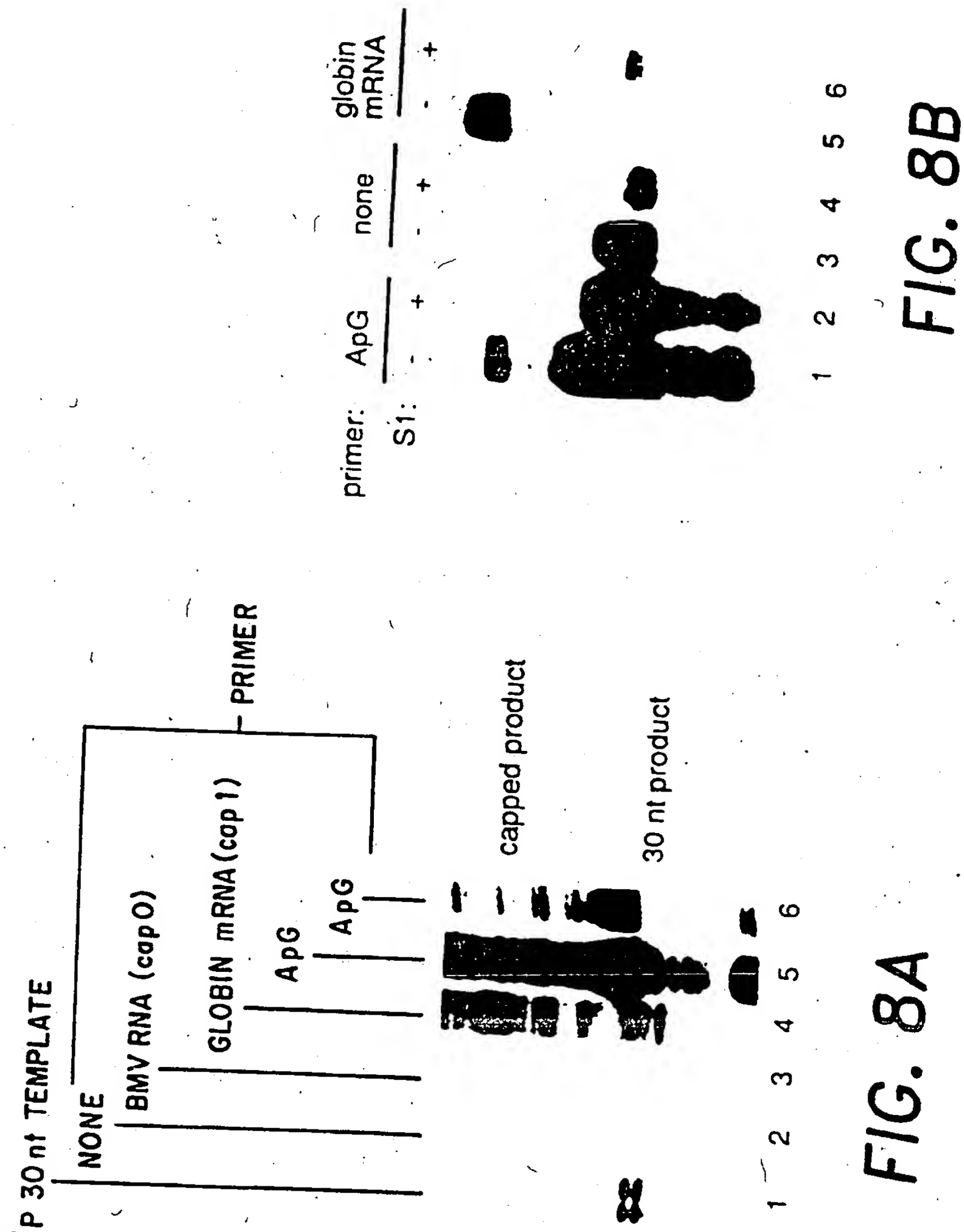

32P 30 nt TEMPLATE
NONE
BMV RNA (cap 0)
GLOBIN mRNA (cap 1)
ApG
ApG
PRIMER
primer: ApG none
S1: - + + -
globin mRNA + -
capped product
30 nt product
1 2 3 4 5 6
1 2 3 4 5 6

FIG. 8A

FIG. 8B

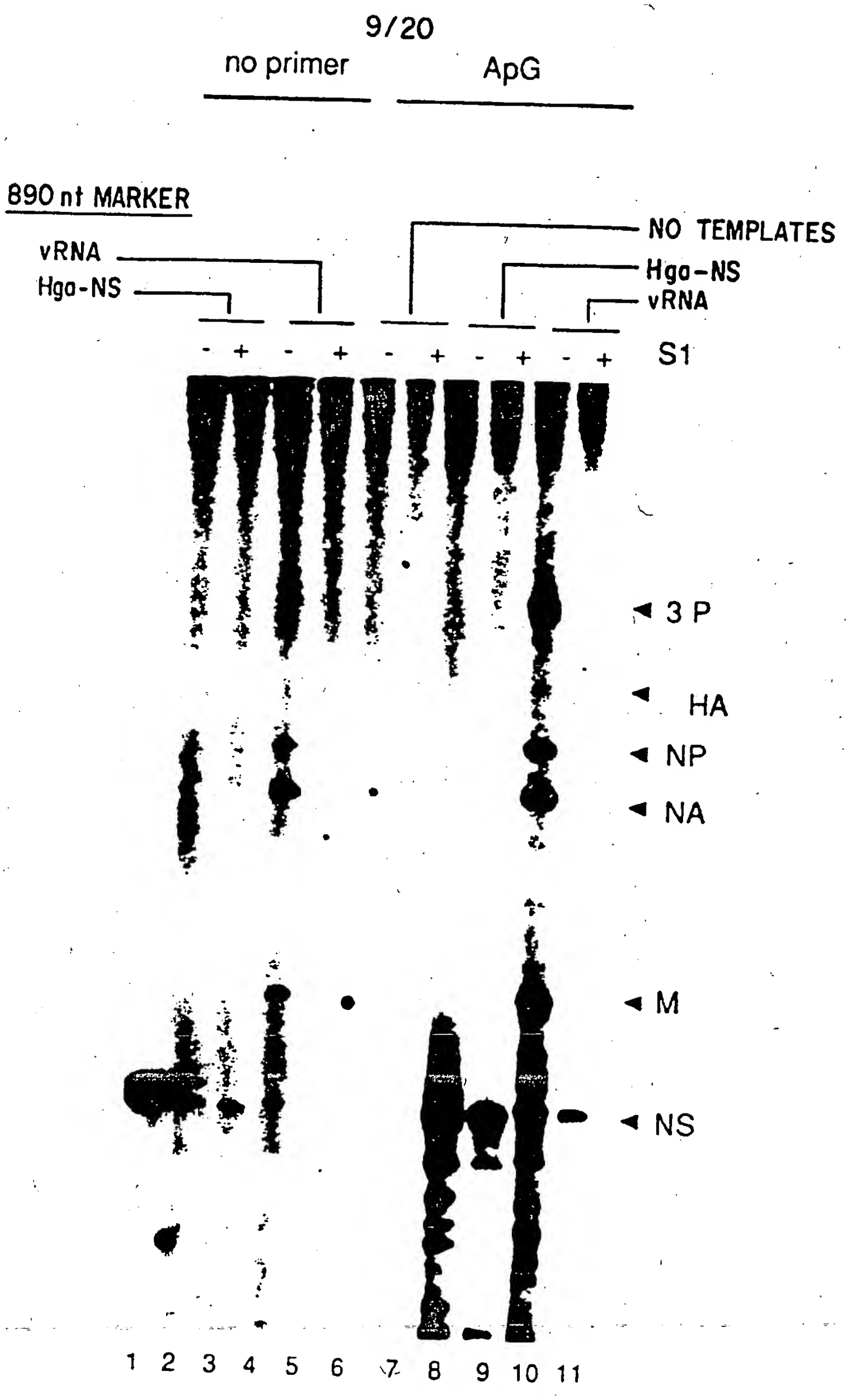

no primer
ApG
890 nt MARKER
NO TEMPLATES
vRNA
Hgo-NS
Hgo-NS
vRNA
- + - + - + - + - + S1
3 P
HA
NP
NA
M
NS
1 2 3 4 5 6 7 8 9 10 11

FIG. 9

FIG. 10

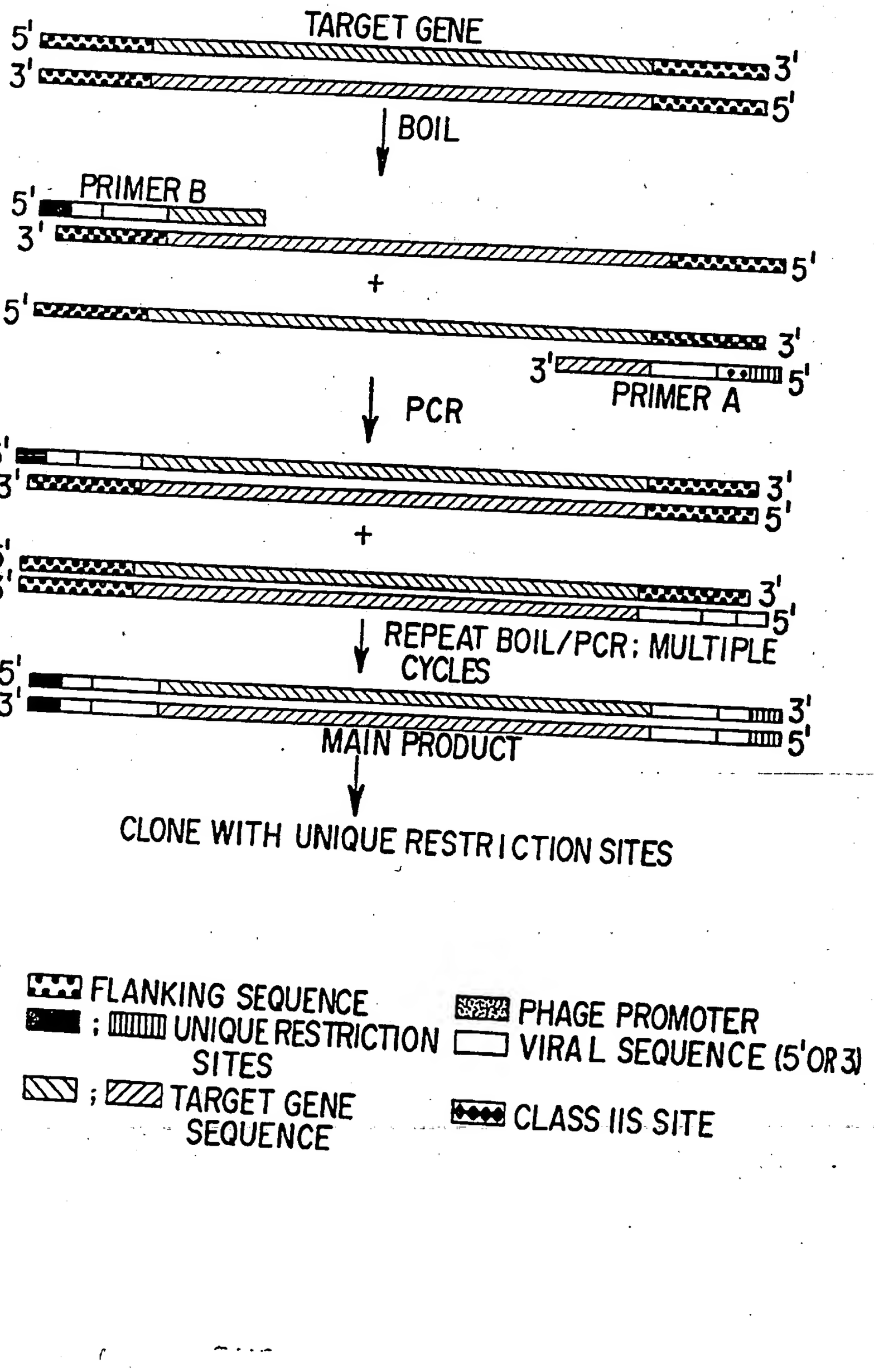

TARGET GENE
5'
3'
3'
5'
BOIL
PRIMER B
5'
3'
5'
+
5'
3'
3'
5'
PRIMER A
PCR
5'
3'
3'
5'
+
5'
3'
3'
5'
REPEAT BOIL/PCR: MULTIPLE CYCLES
5'
3'
3'
5'
MAIN PRODUCT
CLONE WITH UNIQUE RESTRICTION SITES
FLANKING SEQUENCE
; UNIQUE RESTRICTION SITES
; TARGET GENE SEQUENCE
PHAGE PROMOTER
VIRAL SEQUENCE (5' OR 3')
CLASS IIS SITE

FIG. IIA

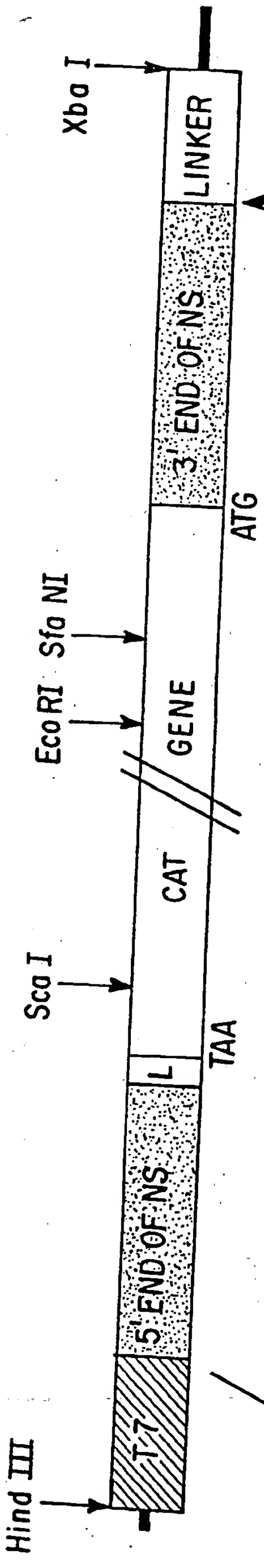
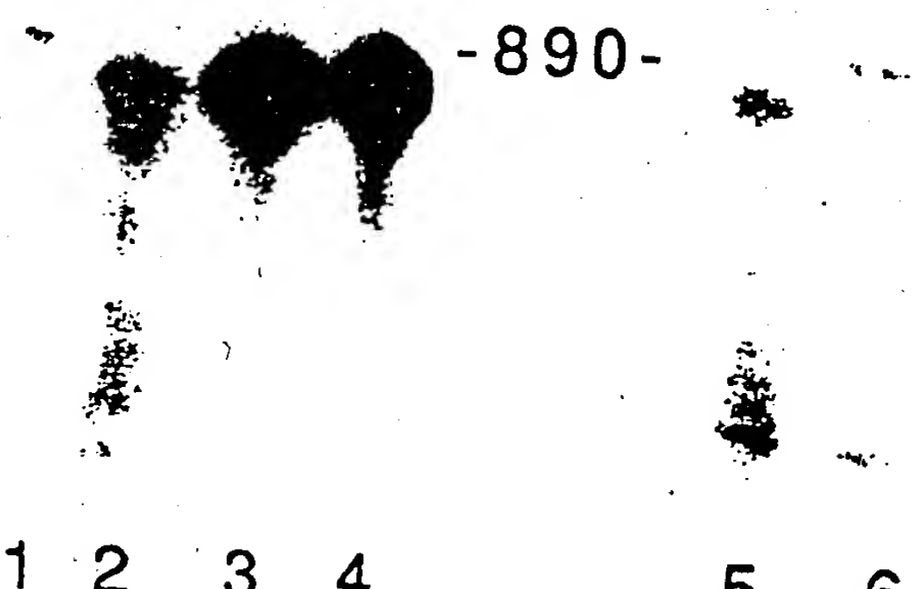


FIG. IIB

5' AGUAGAAACAAGGGUGUUUUUU CAGAUCUA UUACGCCCCGCCC//GUGGUAUACCCAGUGAUUUUUUUCUCCAU U

5' GUUGUCUUUGUCACCCUGCUUUGCU 3'

FIG. 12



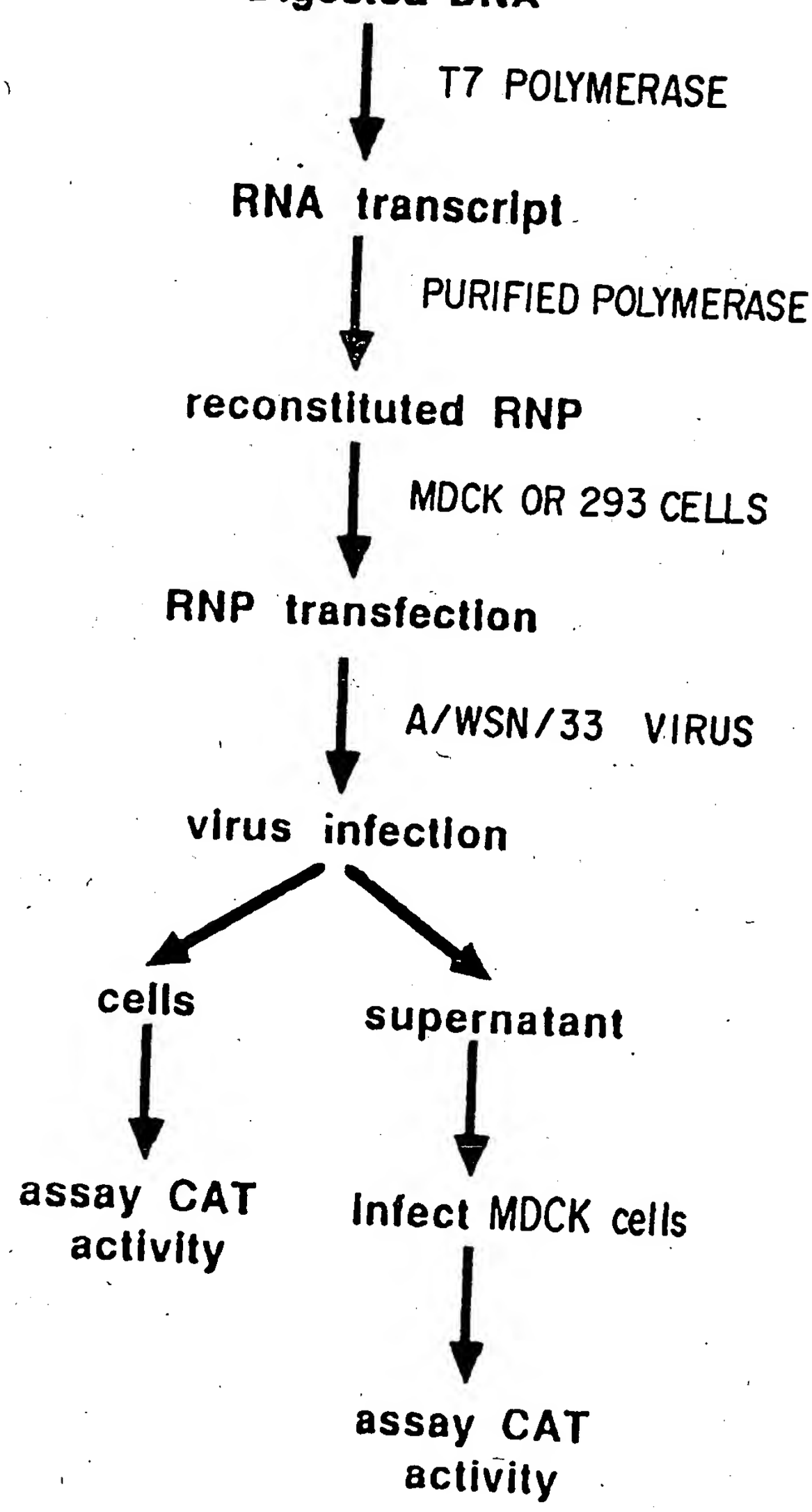

Digested DNA
T7 POLYMERASE
RNA transcript
PURIFIED POLYMERASE
reconstituted RNP
MDCK OR 293 CELLS
RNP transfection
A/WSN/33 VIRUS
virus infection
cells
supernatant
assay CAT activity
Infect MDCK cells
assay CAT activity

FIG. 13

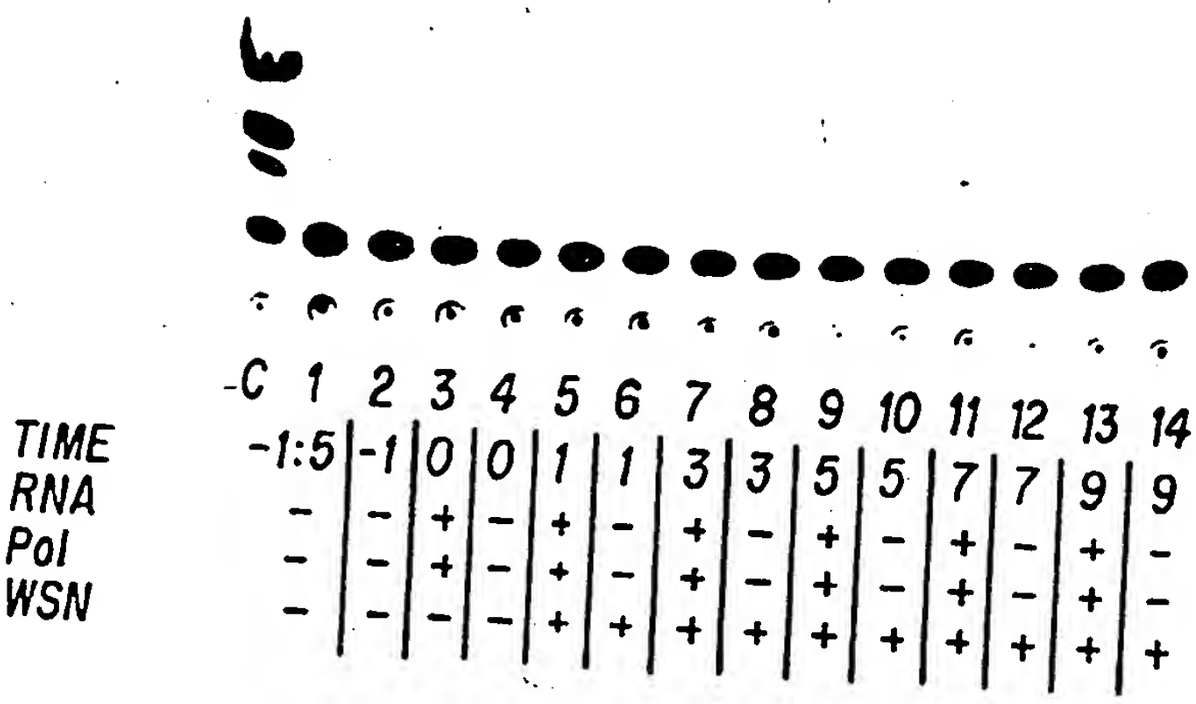


FIG. 14a

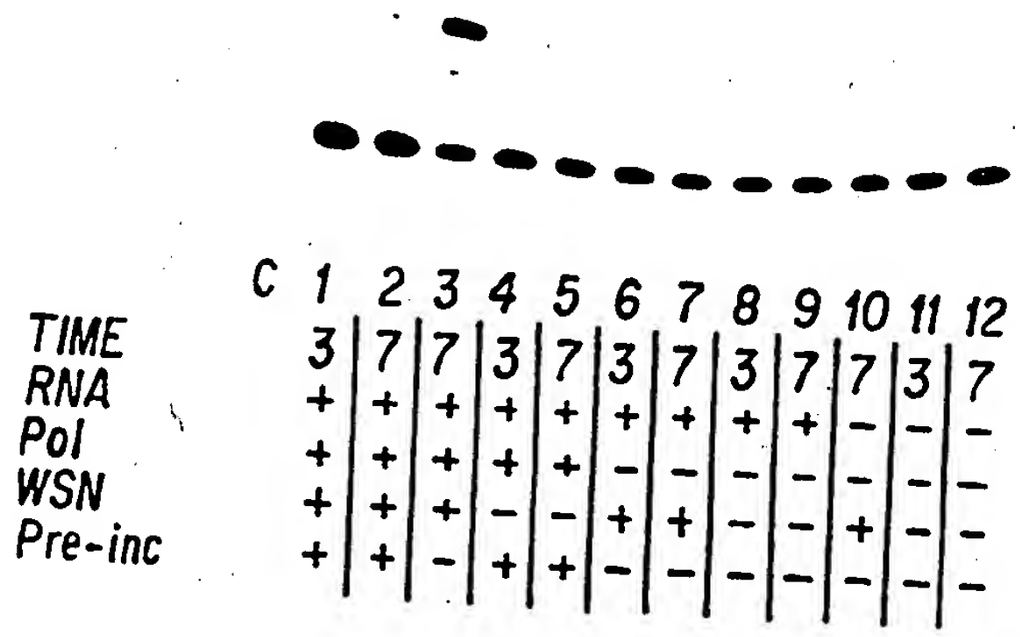


FIG. 14b

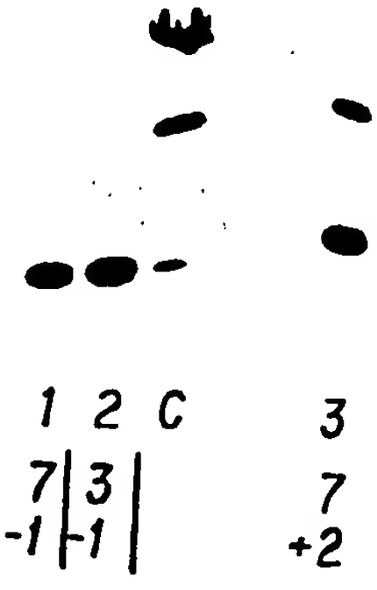
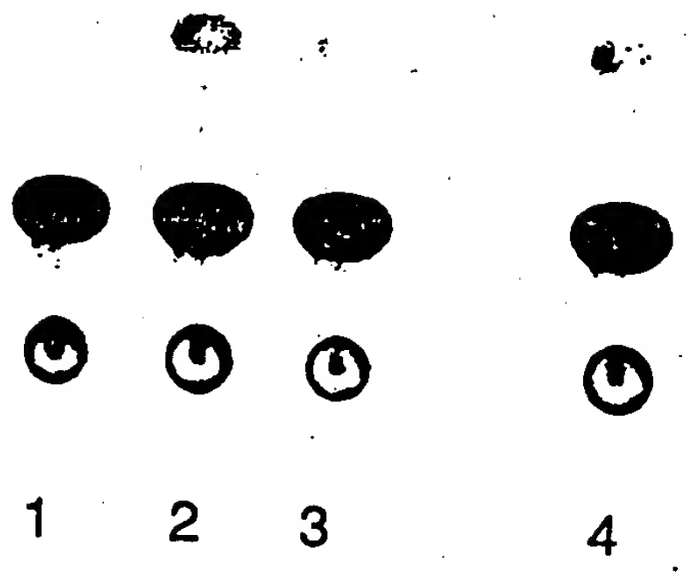


FIG. 14c

FIG. 15



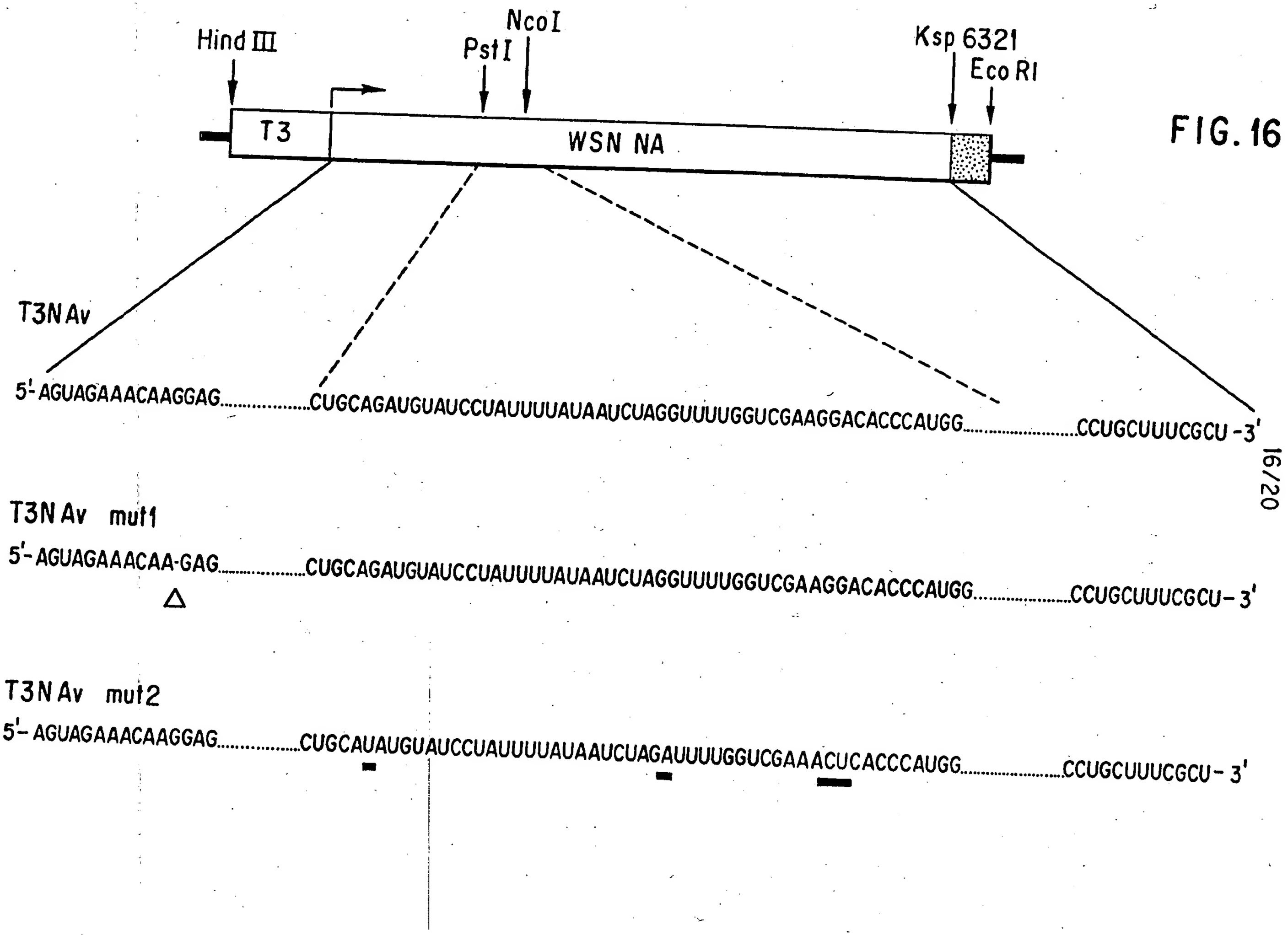

Hind III
PstI
NcoI
Ksp 6321
Eco RI
T3
WSN NA
T3NAv
5'-AGUAGAAACAAGGAG.....CUGCAGAUGUAUCCUAUUUUAUAAUCUAGGUUUUGGUCGAAGGACACCCAUGG.....CCUGCUUUCGCU-3'
T3NAv mut1
5'-AGUAGAAACAA-GAG.....CUGCAGAUGUAUCCUAUUUUAUAAUCUAGGUUUUGGUCGAAGGACACCCAUGG.....CCUGCUUUCGCU-3'
△
T3NAv mut2
5'-AGUAGAAACAAGGAG.....CUGCAUAUGUAUCCUAUUUUAUAAUCUAGAUUUUGGUCGAAACUCACCCAUGG.....CCUGCUUUCGCU-3'

FIG. 16

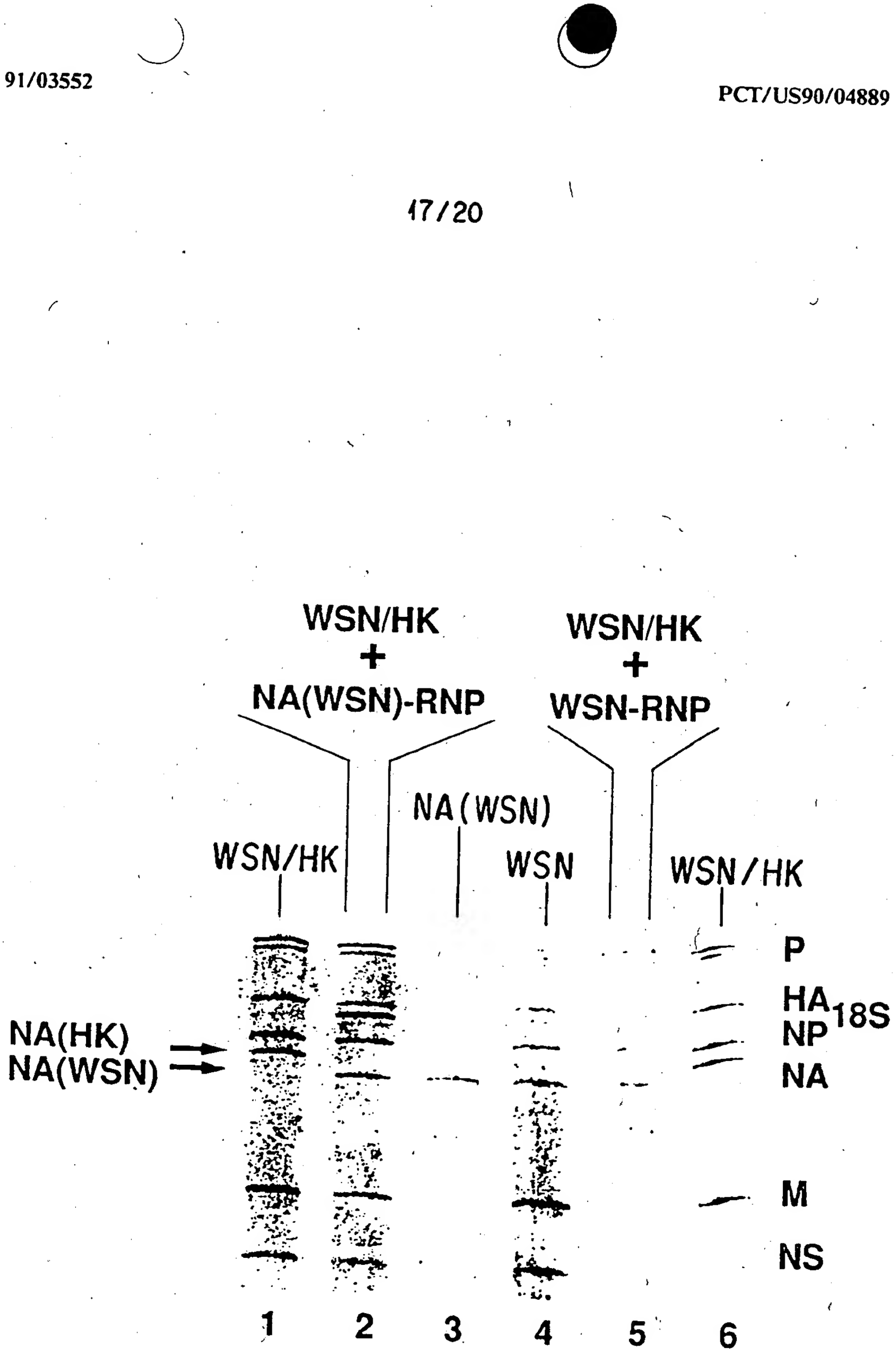

WSN/HK
+
NA(WSN)-RNP
WSN/HK
+
WSN-RNP
WSN/HK
NA(WSN)
WSN
WSN/HK
NA(HK)
NA(WSN)
P
HA
NP
NA
18S
M
NS
1
2
3
4
5
6

FIG. 17

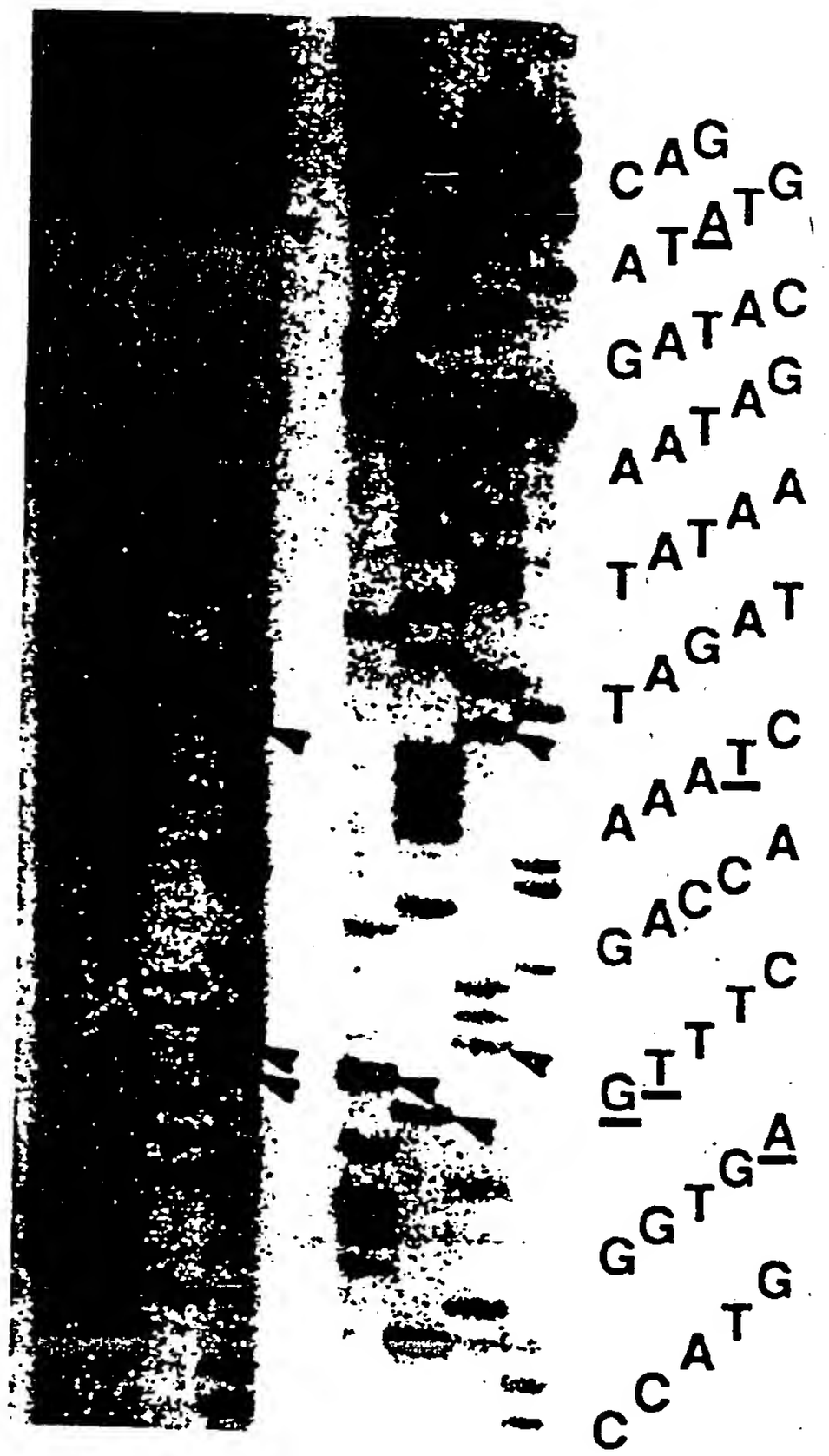

GATC GATC
CAGATGGATACAATAGTATAAGATAAAICGACCAGTTCGGTGAGCCATG

FIG. 18

FIG. 19

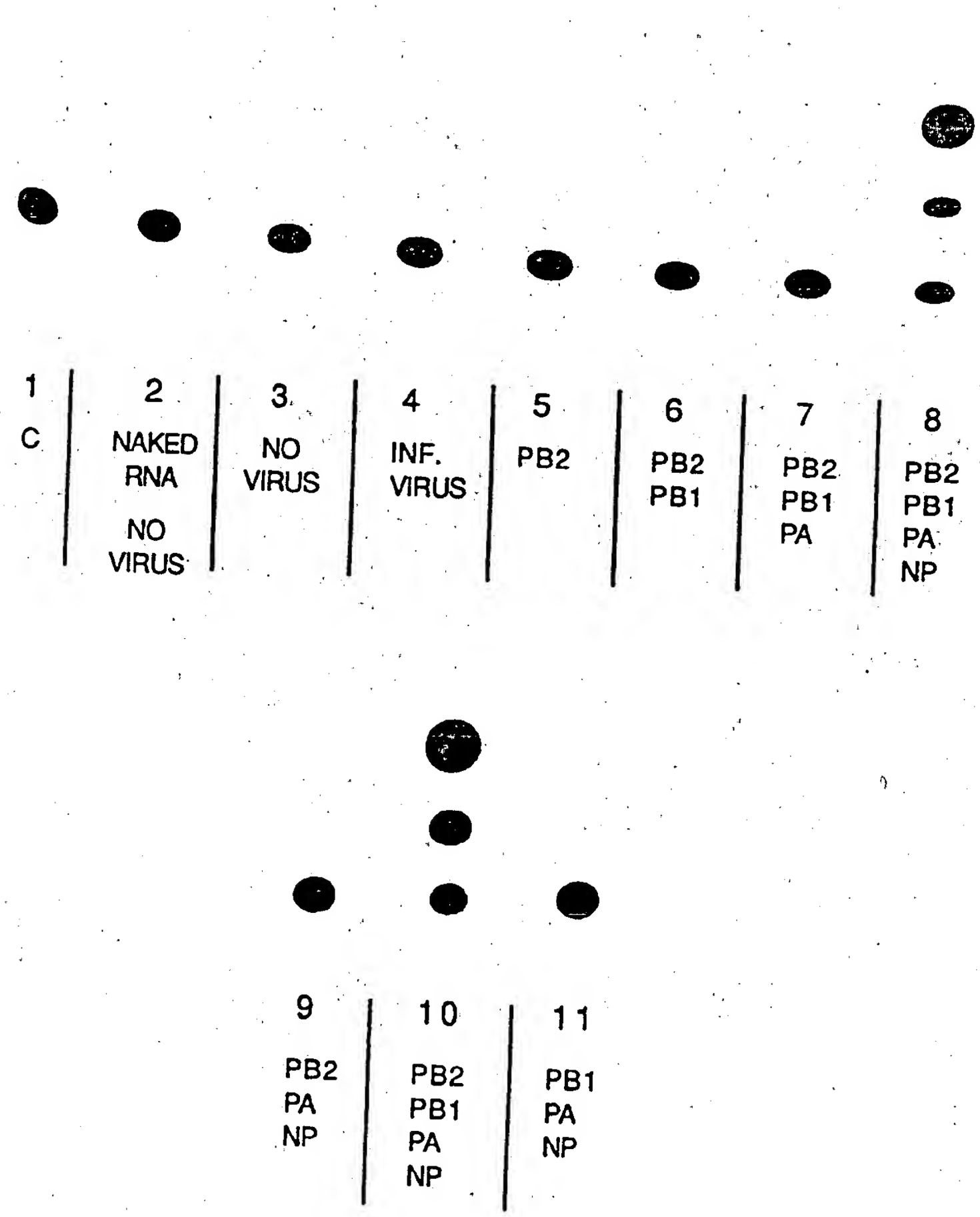

1
C
2
NAKED RNA
NO VIRUS
3
NO VIRUS
4
INF. VIRUS
5
PB2
6
PB2
PB1
7
PB2
PB1
PA
8
PB2
PB1
PA
NP
9
PB2
PA
NP
10
PB2
PB1
PA
NP
11
PB1
PA
NP

FIG. 20a

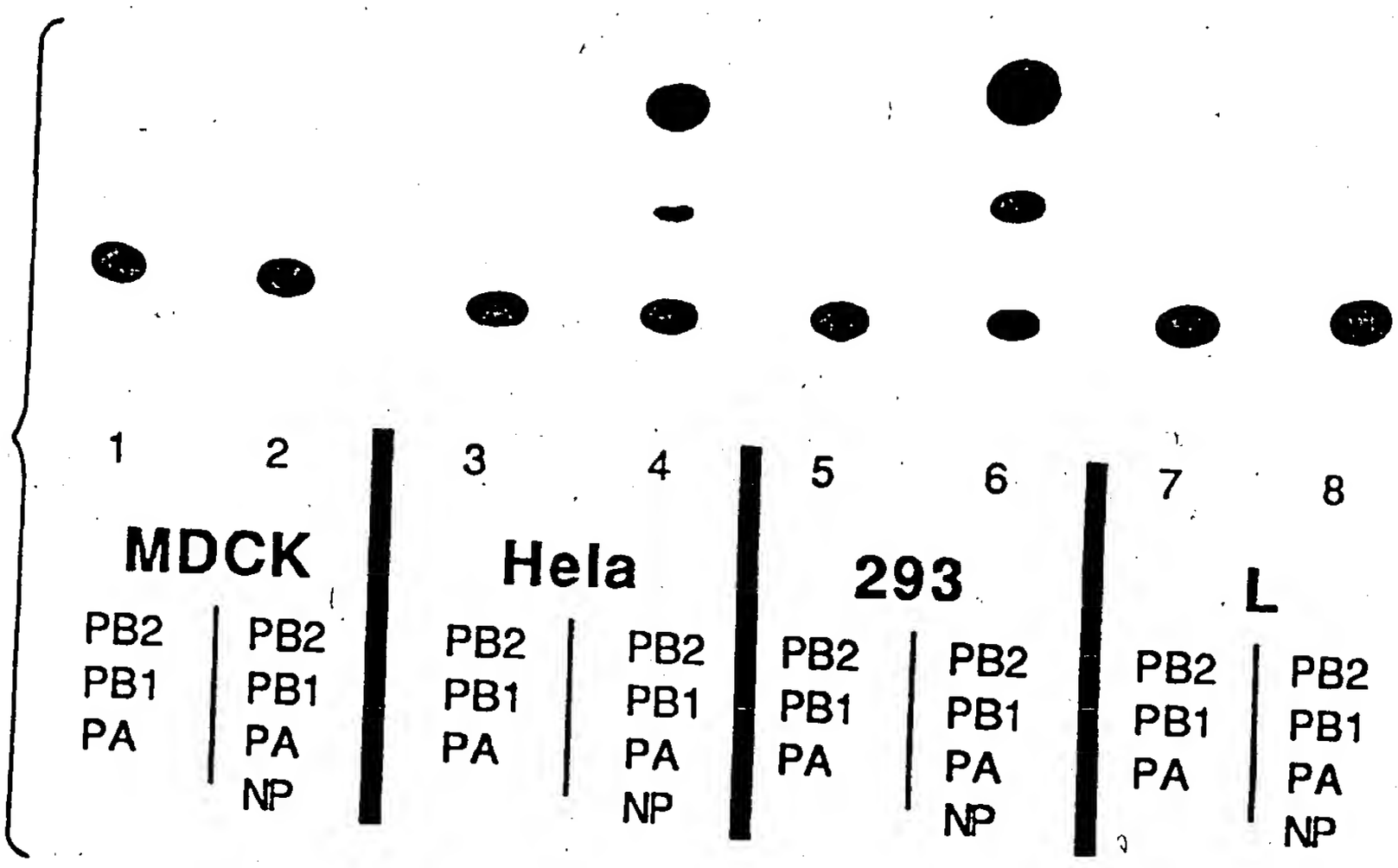


FIG. 20b

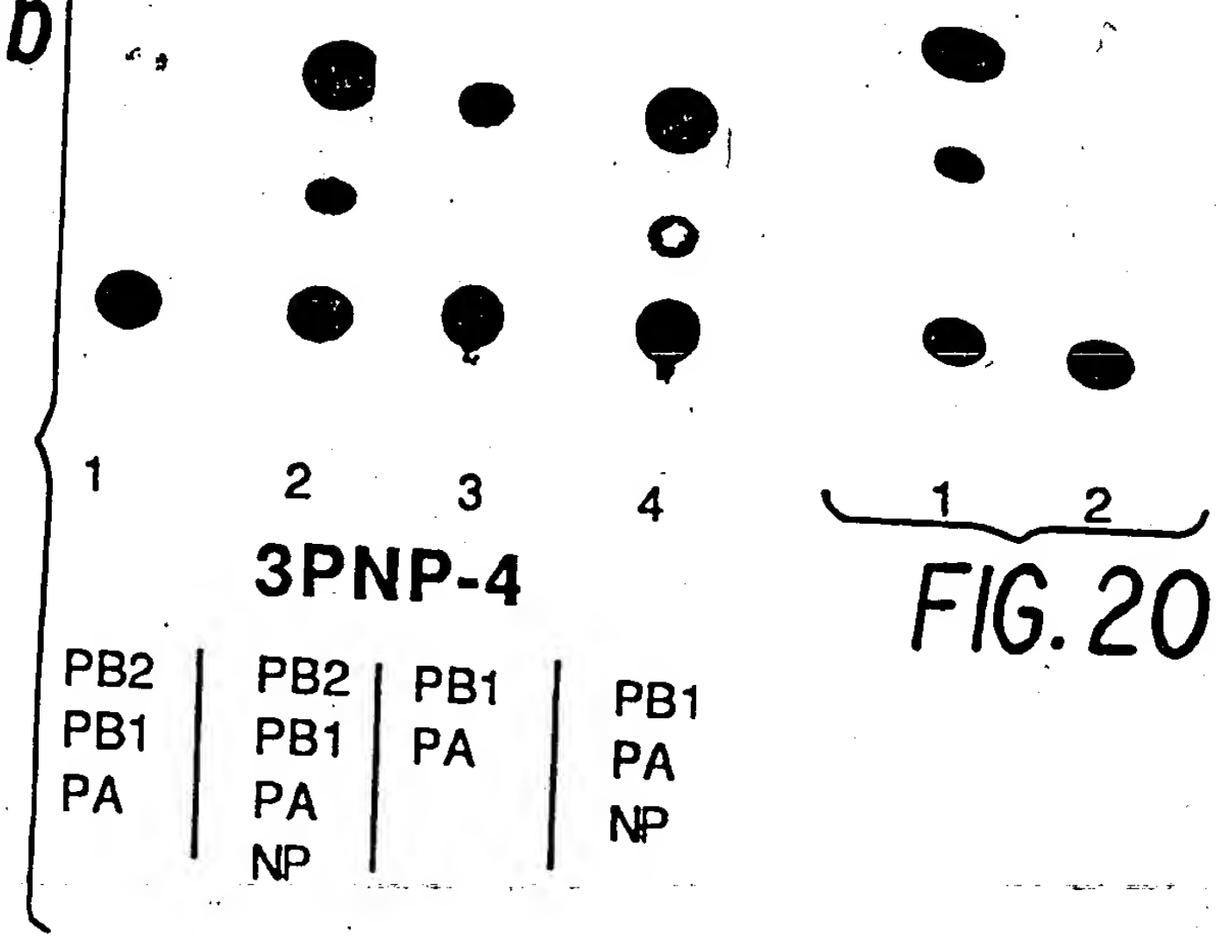


FIG. 20c

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/04889

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ³

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 15/11, 15/86; C12N 7/01; C12N 9/12
U.S. Cl: 435/172.3, 235, 194; 536/27

II. FIELDS SEARCHED

Minimum Documentation Searched ⁴

Classification System	Classification Symbols
U.S. Cl.	435/172.3, 235, 320, 194, 91; 536/27; 935/6, 8, 16, 32, 57

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁶

III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴

Category ⁵	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	Proceedings of the National Academy of Sciences, USA, volume 84, issued November 1987, Hsu et al, "Genomic RNAs of influenza viruses are held in a circular conformation in virions and in infected cells by a terminal panhandle", pages 8140-8144. See entire document.	1-24
Y	Chemical Abstracts, volume 104, issued 1986, Levis et al, "Deletion mapping of Sindbis virus DI RNAs derived from cDNAs defines the sequences essential for replication and packaging", page 137, abstract 162747. See entire document.	1-24

⁵ Special categories of cited documents: ¹⁵

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search ²

28 November 1990

Date of Mailing of this International Search Report ²

09 JAN 1991

International Searching Authority ¹

ISA/US

Signature of Authorized Officer ²⁰

Jeanne M. Mosher for
Mary E. Mosher

Form PCT/ISA/210 (second sheet) (May 1986)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
X	US, A, 4786600 (Kramer et al). 22 November 1988. See abstract, claim 9.	1
Y	Proceedings of the National Academy of Sciences, USA, volume 85, issued November 1988, Szewczyk et al, "Purification, thioredoxin renaturation, and reconstituted activity of the three subunits of the influenza A virus RNA polymerase", pages 7907-7911. See entire document.	1-24
Y	Proceedings of the National Academy of Sciences, USA, volume 85, issued October 1988, Mirakhur et al, "In vitro assembly of a functional nucleocapsid from the negative-stranded genome RNA of a defective interfering particle of vesicular stomatitis virus", pages 7511-7515. See entire document.	1-24
Y	Journal of Biochemistry, volume 104, No.6 issued 1988, Honda et al, "RNA polymerase of influenza virus: role of NP in RNA chain elongation", pages 1021-1026. See entire document.	1-24
Y	Journal of Virology, volume 62, No.7 issued July 1988, Shapiro et al, "Influenza virus replication in vitro: synthesis of viral template RNAs and virion RNAs in the absence of an added primer", pages 2285-2290. See entire document.	1-24
Y	Journal of Molecular Biology, volume 201, issued 1988, Dreher et al, "Mutational analysis of the sequence and structural requirements in brome mosaic virus RNA for minus strand promoter activity", pages 31-40. See entire document.	1-24
Y	Nature, volume 311, 13 September issued 1984, Dreher et al, "Mutant viral RNAs synthesized in vitro show altered aminoacylation and replicase template activities", pages 171-175. See entire document.	1-24

Attachment to PCT/US90/04889

Search terms:

RNA directed RNA polymerase, influenza, vector, template, transcript?, negative strand, minus strand, polymerase binding, virus, satellite, K. Yamanaka, sequenc?, control?; also searched sequences listed in claims and their reverse complements.

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(74) Agent: CULLEN & CO; Level 12, 240 Queen Street, Brisbane, QLD 4000 (AU).

(81) Designated States: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP, KP, KR, KZ, LK, LU, LV, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SK, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: VECTOR TO DELIVER AND EXPRESS FOREIGN GENE

(57) Abstract

A vector for delivering a foreign gene to a target cell for expression of the foreign gene is provided. The vector comprises a (-) sense RNA genome contained within a ribonucleoprotein complex within a virus-like particle constituted from structural proteins of a (-) sense RNA virus. The (-) sense RNA genome includes one or more foreign genes but does not include genes for replication of the (-) sense RNA virus. Methods of preparing the vector are disclosed, as well as pharmaceutical compositions containing the vector, and methods of delivering the expression product of the foreign gene to a target cell.

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VECTOR TO DELIVER AND EXPRESS FOREIGN GENE

TECHNICAL FIELD

This invention relates to a delivery system which can be untilised to deliver and express a foreign gene in eukaryotic cells. In particular, the delivery system can be used to deliver an RNA gene which will direct synthesis of an encoded anti-sense RNA, catalytic RNA, peptide or polypeptide within specific target cells which can be of a selected type. The delivery system can be used *in vitro* to target eukaryotic cells in culture or can be used *in vivo* to deliver a prophylactic or therapeutic agent to specific cells in an animal or human that is diseased or infected or at risk of disease, infection or infestation.

BACKGROUND ART

Hitherto, conventional systems for delivery of therapeutic agents included pharmaceutical dosage forms such as capsules which are made principally of gelatin blends and which contain small amounts of other components such as dyes, plasticisers, preservatives and opaquing agents. These capsules function as a soluble external shell or envelope for delivery of drugs to a required location. Soft capsules are used for liquids while hard capsules are used for delivery of free flowing powders. Microencapsulation techniques are also well known. Other types of pharmaceutical dosage forms have included compressed tablets prepared by compaction of a formulation containing the drug and certain excipients selected to aid the processing and to improve the properties of the drug. These excipients can include binders, disintegrants, fillers, or diluents and lubricants. Film coated tablets are compressed tablets with a film coat applied. An example is an enteric coated tablet which allows the drug to be delivered to the intestines because the coating is insoluble in the stomach. Also known are sustained release tablets which allow release of the drug over a period of time.

In both dosage forms described above, the drugs normally reach the gastro-intestinal tract (GI tract) and diffuse across the gastro-intestinal membrane into the bloodstream. The drug contained in the tablet dosage form will disintegrate in the GI tract prior to entry into the bloodstream

due to the presence of the disintegrant and the capsule dosage form will dissolve prior to the drug entering the bloodstream.

However, a major problem in the delivery of drugs and other macromolecules into cells is the permeability barrier imposed by the plasma membrane. Pharmaceutical dosage forms comprising tablets or capsules are unable to penetrate the permeability barrier, especially in relation to macromolecules which can comprise polypeptides such as toxins, enzymes or antibodies, or polynucleotides such as DNA or RNA.

Various methods have been used for delivery of macromolecules into cells. These include physical treatments such as microinjection, permeabilisation by lytic agents or high voltage electric fields and induced uptake of calcium phosphate or polyethylene glycol co-precipitates. Cell entry by fusion of a delivery vehicle with the cell plasma membrane has been achieved by use of liposomes and reconstituted viral envelopes (RVEs). Live virus vectors and other engineered viral delivery vehicles have also been used. While many of these methods have been useful for *in vitro* delivery of macromolecules to cells in culture, few have been successfully applied to delivery of macromolecules *in vivo*.

Liposomes comprise artificial lipid envelopes which can be generated *in vitro* by condensation of phospholipid into a bilayer membrane which can enclose a soluble macromolecule. Trapping efficiency into liposomes can be as high as 20-30% but the efficiency of delivery of macromolecules is poor, especially *in vivo* where rapid clearance from the bloodstream and high uptake by the liver and spleen present difficulties. In general, liposomes do not allow specific cell targeting but covalent attachment of virus-specific antibodies to the liposome surface has been used to achieve delivery of macromolecules to virus infected cells *in vitro*.

RVEs comprise viral envelopes which have been formed by solubilising intact virus in detergent and reassembling the viral envelope on removal of the detergent. RVEs can be formed in the presence of therapeutic agents including macromolecules which become encapsulated and can be used for drug delivery *in vitro* and *in vivo*. Encapsulation efficiency for macromolecules is lower than for liposomes (3-5%) but delivery efficiency and

cell targeting are enhanced by the presence of viral spike glycoproteins in the RVE membrane. The spike glycoproteins recognise receptors in the plasma membrane of the target cell. Cells which lack the specific receptor are not recognised by the RVEs and so are not targeted for delivery. The spike glycoproteins also contain a fusion domain which enhances fusion of the RVE with the cell membrane. Methods for modifying the target specificity of RVEs by covalent attachment of various ligands to the spike glycoprotein have also been described and the use of genetically engineered chimeric attachment proteins containing specific surface receptor recognition domains has been suggested.

Live virus vectors have been used both *in vitro* and *in vivo* to deliver genes encoding prophylactic and therapeutic agents such as vaccine antigens and interleukins and to effect synthesis of the products in the target cells. A gene encoding the therapeutic agent is engineered into the viral genome and the product is expressed upon infection of target cells.

In live DNA virus vectors, the virus is engineered to contain a foreign gene or genes at a site in the genome which does not inhibit the infectivity of the virus. The virus can also be engineered to have reduced virulence for the target host. After infection of host cells, the virus expresses viral products as well as the foreign product. DNA viruses which have been engineered as live, replicating delivery vehicles include poxviruses herpesviruses, adenoviruses, papovaviruses, parvo-viruses and baculoviruses of insects.

While live replicating virus vectors can be effective and efficient delivery vehicles, they are not usually acceptable for general human or veterinary use because of the risk of causing disease and because of potential environmental risks due to infection of non-target species. DNA viruses can also incorporate integration elements which can modify the host genetic structure and present the risk of inducing tumours and related disorders.

RNA viruses used for delivery of foreign genes to animal cells include retroviruses, alphaviruses, Semliki forest virus, Sindbis virus and influenza virus. Retrovirus vectors are usually constructed by transfection of helper cells with a

DNA molecule which contains the terminal domains (LTRs) and assembly elements (psi region) of a retrovirus and includes the coding region of a foreign gene. The helper cells express retrovirus structural proteins. The transfected DNA molecule is integrated into the DNA of the helper cells and an RNA molecule corresponding to a modified retrovirus genome is expressed. The modified genome including the foreign gene can be assembled into retrovirus-like particles by using the structural proteins expressed in the helper cells. Retrovirus vectors can be used for delivery of foreign genes into cells in the form of RNA which is transcribed into DNA and can be integrated into the host chromosomes and subsequently expressed by the host cell. Although retroviral vectors are a useful laboratory tool and have been used in particular cases for gene therapy, more general use is restricted by concerns that the host genetic structure can be modified resulting in tumours and related disorders.

The alphavirus Sindbis has been used as a delivery vehicle for expression of foreign genes in animal cells *in vitro*. Sindbis virus causes an acute febrile illness in humans and is transmitted by biting insects. Unlike retroviruses, the virus does not synthesise DNA or induce tumours in infected animals. Sindbis virus vectors have been constructed by deleting genes encoding the capsid structural proteins from the genome and substituting a foreign gene. However, as a (+) sense RNA virus, Sindbis does not carry a viral RNA transcriptase as a structural component of the particle. Efficient expression of the foreign gene in target cells requires expression of the viral replicase and transcriptase components which are encoded in the 5' two-thirds of the genome. Thus, the use of Sindbis virus expression vectors for delivery of therapeutic agents *in vivo* has three disadvantages: (i) the gene encoding the therapeutic agent cannot be delivered and expressed without prior expression of some viral proteins (replicase and transcriptase proteins); (ii) a limited amount of cloning capacity, approximately 3475 nucleotides, remains for insertion of foreign genes in the absence of infectious helper virus; and, (iii) the vector may become contaminated with wild type infectious virus due to recombination between the vector and

helper virus during vector preparation.

An influenza virus has been described in which the influenza A virus NS gene was replaced by a foreign indicator gene. When mammalian cells were transfected with the foreign gene, purified influenza virus polymerase complex and helper virus, recombinant virus particles were formed. As with the Sindbis virus vectors, the foregoing recombinant influenza virus vector has the disadvantages that the vector remains capable of expressing influenza proteins in the target cell and can revert to virulence by recombination with live virus.

Pattnaik and Wertz *(Proc. Natl. Acad. Sci. USA 88,* 1379-1383 (1991)) have described infectious defective interfering (DI) vesicular stomatitis virus particles produced by infecting cells with DI particles where the cells harboured vectors for the expression of all five vesicular stomatitis virus proteins. Such particles are not suitable for delivering a foreign gene to a target cell because of the infectivity of the particles.

Other recombinant (-) sense RNA virus particles have been described by Park *et al. (Proc. Natl. Acad. Sci. USA 88,* 5537-5541 (1991)) and Collins *et al. (Proc. Natl. Acad. Sci. USA 88,* 9663-9667 (1991)). These publications respectively describe Sendai virus particles and respiratory syncytial virus (RSV) particles which package a foreign gene. In both instances however, formation of the recombinant virus particles was dependent on co-transfection with live Sendai virus or RSV particles resulting in the production of infectious virus particles. The methods described by Park *et al.* and Collins *et al.* are thus not suitable for delivering a foreign gene to a target cell because of the risk posed by viral infection.

In vivo delivery of therapeutic proteins to keratinocytes using a retrovirus vector is known as is a drug delivery virion in a retrovirus envelope which contains a protein drug sequence useful as an anti-leukaemia and anti-tumour agent. Poxvirus expression systems for delivery of vaccine antigens and a system for delivery of genetic material into brain cells using a virus vector have also be described.

While the abovementioned prior art make it clear that viral vector delivery systems for foreign genes coding for a

protein therapeutic or prophylactic agent are not new, there remain difficulties associated with their general use *in vivo*. A live virus vector is not completely safe as it can revert to virulence, cause undesirable effects in the host or can be spread to non-target hosts. Non-replicating retroviral vectors present risks associated with alteration to the host chromosomes that can result in tumours and other available RNA virus delivery systems are limited in the scope of their application and have the disadvantage that they will express some viral products as well as the foreign gene.

The prior art includes descriptions of particles, referred to as virus-like particles (VLPs), which can be constructed by expressing viral structural genes in cultured eukaryotic cells. The procedure has been used to construct synthetic VLPs of several animal and human viruses. For example, the insertion of the complete polycistronic mRNA of poliovirus in the baculovirus polyhedrin gene has been reported. Insect cells infected with the recombinant baculovirus synthesised and processed the poliovirus polyprotein and generated "empty" poliovirus-like particles (VLPs). These synthetic "empty" capsids contained no RNA and were not infectious but were in some aspects similar to the complete virus. Similar methods have been used to construct core-like particles (CLPs) and VLPs of several other viruses including bluetongue, hepatitis B virus and bovine immunodeficiency virus. To date, the particles formed by this method have been generated by protein-protein interactions alone and have not contained defined molecules of nucleic acid (RNA or DNA). Hence, the technology has not yet been applied to the generation of VLPs of (-) sense RNA viruses which appear to require an RNA genome or genome fragment for initiation of the particle assembly process.

It has also been demonstrated that infectious viral particles can be recovered from cDNA clones representing the entire genome of some viruses. In this method cDNA is inserted into plasmid vectors containing promoters operative in eukaryotic cells. Transfection of eukaryotic cells with such vectors results in the production of infectious virus. This general approach has been used in relation to a number of viruses of humans, animals and plants including poliovirus,

Sindbis virus and brome mosaic virus. However, the method has only been applied to some DNA viruses and (+) sense RNA viruses with a genome that can function directly as an mRNA.

SUMMARY OF THE INVENTION

It is the object of this invention to provide an effective and completely non-infectious system for delivery of foreign genes to animal or human cells. The foreign gene will be in the form of a (-) sense RNA.

According to a first embodiment of this invention, there is provided a vector for delivering a foreign gene to a target cell for expression of said foreign gene, said vector comprising a (-) sense RNA genome contained within a ribonucleoprotein complex within a virus-like particle constituted from structural proteins of a (-) sense RNA virus, wherein said (-) sense RNA genome includes one or more foreign genes but does not include genes for replication of said (-) sense RNA virus.

According to a second embodiment of this invention, there is provided a method of preparing a vector for delivering a foreign gene to a target cell for expression of said foreign gene, said vector comprising a (-) sense RNA genome contained within a ribonucleoprotein complex within a virus-like particle constituted from structural proteins of a (-) sense virus, wherein said (-) sense RNA genome includes one or more foreign genes but does not include genes for replication of said (-) sense RNA virus, which method comprises the following steps:

i) preparing an expression vector incorporating a DNA molecule which contains DNA corresponding to said (-) sense RNA genome;

ii) introducing the expression vector prepared in step (i) into a eukaryotic host cell together with DNA for the expression of proteins for the formation of virus-like particles;

(iii) culturing the eukaryotic host cells under conditions which allow expression of said (-) sense RNA genome and said proteins, and incorporation of said (-) sense RNA genome into virus-like particles; and

(iv) harvesting said virus-like particles from the eukaryotic cell culture of step (iii).

According to a third embodiment of this invention,

there is provided a pharmaceutical composition comprising a pharmaceutically acceptable carrier, diluent, adjuvant and/or excipient together with a vector for delivering a foreign gene to a target cell for expression of said foreign gene, said vector comprising a (-) sense RNA genome contained within a ribonucleoprotein complex within a virus-like particle constituted from structural proteins of a (-) sense virus, wherein said (-) sense RNA genome includes one or more foreign genes but does not include genes for replication of said (-) sense RNA virus, and wherein said ribonucleoprotein complex includes a polymerase for synthesis of (+) sense RNA from said (-) sense RNA.

According to a fourth embodiment of this invention, there is provided a method of delivering the expression product of a foreign gene to a target cell, said method comprising contacting said target cell with a vector according to the first embodiment and co-transforming or co-transfecting said cell with a vector which provides an RNA-dependent RNA polymerase activity.

According to a fifth embodiment of this invention, there is provided a method of delivering the expression product of a foreign gene to a target cell, said method comprising contacting said target cell with a vector according to the first embodiment which further comprises within said ribonucleoprotein complex a polymerase for synthesis of (+) sense RNA from said (-) sense RNA genome.

According to a sixth embodiment of this invention, there is provided a method of delivering the expression product of a foreign gene to cells of a tissue of a mammalian subject, said method comprising administering to said subject a vector according to the first embodiment which further comprises within said ribonucleoprotein complex a polymerase for synthesis of (+) sense RNA from said (-) sense RNA genome, or a pharmaceutical composition according to the third embodiment.

The (-) sense RNA genome of the vector of the first embodiment incorporates terminal fragments of the genome of a (-) sense RNA virus to facilitate packaging of the genome into the virus-like particles (VLPs). The VLPs contain the necessary viral proteins to target and enter specific cells and preferably

contains a protein to synthesize (+) sense RNA (ie. mRNA) transcripts of the foreign gene. The expression product of the foreign gene can be a peptide or polypeptide. The peptide expression product can be a biologically active molecule, specific therapeutic agent or immunogen. Similarly, the polypeptide expression product can be a biologically active protein, specific therapeutic agent or immunogen. The VLP vector also permits delivery of anti-sense RNA or catalytic RNA to a target cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of the method of preparing the VLP vectors of the invention and the use of the VLP vectors for delivering a gene product to a target cell.

Figure 2 is a representation of a DNA construct comprising 5' and 3' domains, ribozyme domains R1 and R2 and a filler domain into which a foreign gene can be inserted at a preferably unique restriction endonuclease site such as the *NcoI* site shown.

Figure 3 depicts a process for preparing a genome construct comprising 5' and 3' domains, ribozyme domains and a filler domain.

Figure 4 depicts a process for preparing VLP particles from the genome construct resulting from the process depicted in Figure 3. The following abbreviations are used for restriction endonuclease sites: B, *Bam*HI; E, *Eco*RI; P, *Pst*I; and S, *Sma*I.

Figures 5a to 5d depict typical steps in the construction and cloning of a chimeric G protein gene. Figure 5a depicts the construction of an "anchor" gene fragment; Figure 5b the construction of a "donor" gene fragment; Figure 5c the construction of a chimeric G protein gene; and, Figure 5d depicts the cloning of the chimeric G protein gene.

Figure 6 is a schematic representation of the construction of baculovirus transfer vectors harbouring TB2-CAT genome constructs. The position of the polyhedron gene promoter and the direction of transcription therefrom in pAcYM1 and derivatives are indicated by the symbol "P" and the adjacent arrow respectively. The following abbreviations are used for restriction endonuclease sites: B, *Bam*HI; N, *Nco*I; and V, *Eco*RV

Figure 7 presents nucleotide sequences of the CAT and

CAT3 PCR products with indication of the positions of the terminal and internal *NcoI* restriction enzyme sites. The CAT3 sequence is indicated in full with nucleotide differences in the CAT sequence shown above the sequence. The CAT sequence: (1) has a nucleotide modification at position 357 (T substituted for A) resulting in an amino acid change from Ile to Leu; and, (2) does not contain the rabies virus transcription termination/ polyadenylation sequence CATG[A], immediately following the CAT3 gene translation stop codon (TAA).

BEST MODE AND OTHER MODES OF CARRYING OUT THE INVENTION

In the description of the invention set forth below, the following abbreviations are used:

CAT	chloramphenicol acetyl transferase
DIG	digoxigenin
EDTA	ethylenediaminetetraacetate
IPTG	isopropylthio-β-D-galactoside
LMT	low melting temperature
PCR	polymerase chain rection
TD	a solution of 0.8 mM tris-HCl (pH 7.4), 150 mM NaCl, 5 mM KCl and 0.7 mM $Na_2 HPO_4$ which is adjusted to pH 7.5 with HCl and autoclaved
VLP	virus-like particle
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

The term "foreign gene" is used in the following description and claims to denote a gene that is not normally present in the specific cells targeted by the VLP vector or if normally present in the specific cells, is not expressed at the level attainable after delivery of the foreign gene by the VLP vector of the invention.

In alternative nomenclatures used in relation to (-) sense RNA viruses, the M1, P and NS genes and respective expression products are equivalent as are the M2 and M genes and respective expression products.

A preferred process according to this invention, by which VLP vectors can be prepared and utilised to deliver the expression product of a foreign gene to a target cell, inlcudes the following steps:

(i) constructing a DNA molecule corresponding to a

modified genome or genome fragment of a (-) sense RNA virus where the DNA molecule contains a sequence corresponding to the coding region of a foreign gene or the coding regions of two or more foreign genes;

(ii) inserting the DNA molecule prepared in step (i) into an expression vector suitable for transfection of eukaryotic cells;

(iii) transfecting a eukaryotic cell with the recombinant expression vector prepared in step (ii) and simultaneously transfecting the same eukaryotic cell with vectors which express structural proteins of the (-) sense RNA virus and optionally with a vector for the expression of a protein with RNA-dependent RNA polymerase activity;

(iv) obtaining from the cell transfected in step (iii) virus-like particles (VLPs) consisting of a modified genome or genome fragment transcribed from the DNA molecule constructed in step (i), complexed with the viral proteins to form a ribonucleoprotein complex enclosed within a lipid envelope; and

(v) contacting a target cell with the VLPs produced in step (iv) to deliver the foreign gene expression product to the cells or preparing a composition for delivering the VLPs to target cells of tissue of an animal to deliver the foreign gene expression product to those cells.

Advantageously, the structural proteins and protein having RNA-dependent RNA polymerase activity referred to in step (iii) include those with similar functions to the L protein, G protein, N protein, M1 protein and M2 protein of rabies virus. The G protein can be a chimeric G protein incorporating a modified external domain. The VLP formed in step (iv) will thus consist of the modified genome or genome fragment transcribed from the DNA molecule constructed in step (i), complexed with the L protein and M1 protein and surrounded by a sheath of N protein in a ribonucleoprotein complex which is surrounded by an internal matrix comprising the M2 protein and enclosed within a lipid envelope including the G protein (or chimeric G protein incorporating a modified external domain).

The process is not limited to rabies virus however and the structural proteins, protein having RNA-dependent RNA polymerase activity and subgenomic (-) sense RNA fragments can

be obtained from any (-) sense RNA virus having either a segmented or non-segmented genome. Such viruses include, but are not limited to, viruses from the following families: Orthomyxoviridae, Paramyxoviridae, Rhabdoviridae, Bunyaviridae, Arenaviridae and Filoviridae. The preferred viruses are viruses from the rhabdovirus and paramyxovirus genera.

A schematic representation of the process described in the preceding paragraphs is presented in Figure 1.

The DNA molecule referred to in step (i) above typically comprises domains containing DNA sequences corresponding to 5' terminal and 3' terminal non-coding regions of the particular (-) sense RNA viral genome in addition to the sequences corresponding to the coding regions of the one or more foreign genes. Preferably, the 5' and 3' domains are derived from the sequences of the 5' and 3' non-coding regions of the genome of a rhabdovirus or paramyxovirus.

Advantageously, the DNA molecule includes domains encoding ribozymes. The ribozyme domains can be constructed from any of the known ribozyme structures, some of which have been described by Haseloff and Gerlach (*Nature 334*, 585-591 (1988)). The ribozyme domains will be active during step (iii) of the above process and will ensure that the (-) RNA transcript expressed in eukaryotic cells will have a structure suitable for assembly of VLPs.

The foreign gene contained within the DNA construct can be the complete coding region of a selected foreign polypeptide including initiation and termination codons or can be a fragment of a gene corresponding to a functional domain or domains of a polypeptide. The polypeptide encoded by the foreign gene can be an immunogen, a therapeutically or biologically active peptide or polypeptide, or an engineered protein such as an antibody-like molecule. Alternatively, the foreign gene can encode anti-sense RNA or catalytic RNA directed against an intracellular RNA molecule. Multiple foreign genes can be inserted in tandem. A restriction enzyme site, *NcoI* for example, is advantageously included in a generic DNA construct to facilitate insertion of the selected foreign gene or genes to generate the DNA molecule.

The DNA molecule can also include a filler domain

comprising sequences of viral or other origin to give the construct sufficient length to be efficiently packaged in VLPs. The filler domain can constitute any nucleotide sequence that has characteristics which will allow the formation of VLPs. Preferably the filler domain will constitute a fragment derived from a portion of the L protein coding region of a rhabdovirus or paramyxovirus which is adjacent to the 5' terminal non-coding region of the (-) RNA genome. The filler domain will ensure that the genome to be expressed in step (iii) will be of sufficient size to allow formation of VLPs. That size is preferably greater than about 1000 nucleotides.

Advantageously, the DNA molecule incorporates cohesive ends suitable for insertion of the molecule at selected restriction enzyme sites of plasmid vectors.

In relation to step (i) of the process, a typical DNA construct is illustrated in Figure 2.

A DNA construct suitable for carrying a foreign gene is described in international application No. PCT/AU92/00363 (WIPO publication No. WO 93/01833), the entire disclosure of which is incorporated herein by cross-reference. That construct, TB-2, after incorporation into a eukaryotic expression vector as described in step (ii) above and used as described in steps (iii) to (v) above, allows the formation of rabies VLPs. Inclusion of a foreign gene or gene(s) at the *NcoI* site of the TB-2 construct permits construction by steps (i) to (v) above of a rabies VLP which can be used as a vector for delivery of the foreign gene into a eukaryotic cell for expression of the gene in that cell.

In TB-2, the 5' and 3' domains are derived from the known nucleotide sequence of the 5' and 3' terminal regions of the genome of rabies virus (PV and CVS strains). The R1 domain is designed to target a site within the (-) RNA transcript of the TB-2 DNA construct. The R1 ribozyme in the transcript will cleave the RNA to ensure that extraneous parts of the transcript are removed so that the 5' terminus of the transcript corresponds to, or approximates, that of the 5' terminus of the rabies virus genome. Similarly, the R2 ribozyme domain is designed to target a site within the (-) RNA transcript of the TB-2 DNA construct. The R2 ribozyme will cleave the RNA to

ensure that extraneous parts of the 3' region of the transcript (including the R2 domain) are removed so that the 3' terminus of the transcript approximates that of the 3' terminus of the rabies virus genome. The filler domain in the TB-2 construct is derived from the known nucleotide sequence of a 1167 nucleotide region at the 5' end of the rabies virus (PV strain) L protein gene. The TB-2 construct also includes an *NcoI* site at which any selected foreign gene or genes can be inserted. The preparation of a DNA molecule comprising a modified genome of a (-) sense RNA virus is illustrated in Figure 3 using, as an example, the TB-2 DNA molecule derived from the rabies virus genome. Use of such a molecule for the preparation of VLPs is illustrated in Figure 4.

According to the process illustrated in Figure 3, TB-2 DNA is constructed from 3 fragments (Fragment A, Fragment B, and Fragment C in Figure 3). Fragment A incorporates the 5' domain and R1 domain of TB-2 and can be prepared from overlapping complementary oligonucleotides. Suitable oligonucleotides are PJW.5R1A and PJW.5R1B, the sequences of which, together with other oligonucleotides suitable for use in other steps of the procedure, follow:

PJW.5R1A 5'-TACGTCACGCTTAACAAATAAACAACAAAAATGAGAAAAACAATCAAACA-ACTAGAGGTTCAGATTTAAG-3'

PJW.5R1B 5'-TACGTTTCGTCCTCACGGACTCATCAGACGCTTAATGAAAAAAACAAGAT-CTTAAATCTGAACCTCTAGT-3'

PJW.3R2A 5'-CATGGTAGGGGTGTTACATTTTTGCTTTGCAATTGACGCTGTCTTTTTCT-TCTCTGGTTTTGTTGTTAAGCGTC-3'

PJW.3R2B 5'-TTAAGCGTTTCGTCCTCACGGACTCATCAGACCGGCGAAAACACATCGCC-GGTGACGCTTAACAACAAAACCA-3'

PJW.L2R 5'-AGAGTGATAGATTTTGACTGA-3'

PJW.L4R 5'-AAATACATCACACAAGAGTCT-3'

Oligonucleotides PJW.5R1A and PJW.5R1B are annealed and end-filled using T4 DNA polymerase to produce a blunt-end double-stranded DNA molecule of the required nucleotide sequence which can then be cloned into, for example, the *SmaI* site of a suitable plasmid vector such as pBluescript IIKS+. The DNA can then be excised from the vector by using suitable restriction enzymes, *BamHI* and *EcoRI* for example, to generate the required fragment with cohesive ends in the required orientation

(Fragment A, Figure 3).

Fragment C incorporates the 3' domain, R2 domain and the foreign gene insertion site (*Nco*I site) of TB-2 and can be constructed from overlapping complementary oligonucleotide primers PJW.3R2A and PJW.3R2B. By a similar procedure to that described for the construction of fragment A, the oligonucleotides are annealed and end-filled using T4 DNA polymerase to produce a blunt-end double-stranded DNA molecule of the required nucleotide sequence which can be similarly cloned into, for example, the *Sma*I site of a vector such as pBluescript IISK+. The DNA can then be excised from the vector by using suitable restriction enzymes such as *Bam*HI and *Pst*I, to generate the required fragment with cohesive ends in the required orientation (Fragment C, Figure 3).

Fragment B incorporates the filler domain and can be constructed, for example, from the rabies virus (PV strain) genome using primer PJW.L2R (above) and reverse transcriptase to prepare a single-stranded cDNA copy of the required portion of the rabies L protein gene and then by using primers PJW.L2R and PJW.L4R (above) and the polymerase chain reaction (PCR) to amplify a double-stranded DNA molecule of the required nucleotide sequence. The DNA molecule can then be cloned into, for example, the *Sma*I site of a suitable plasmid vector such as pUC8. The DNA can then be excised from the vector using suitable restriction enzymes, *Eco*RI and *Pst*I for example, to generate the required fragment with cohesive ends in the required orientation (Fragment B, Figure 3).

The TB-2 DNA construct can then be assembled by ligation of Fragment A, Fragment B and Fragment C with T4 DNA ligase to join the cohesive ends in the required orientation (Figure 3).

For the production of rabies VLPs, the TB-2 DNA construct is inserted into a vector for synthesis of (-) sense RNA. Advantageously, the (-) sense RNA is synthesised in an insect cell using a baculovirus expression vector. As shown in Figure 4, the TB-2 construct is inserted into a baculovirus transfer vector such as pAcUW31 at the *Bam*HI site to form pAcUW31.TB2. Recombinant baculovirus capable of expressing TB-2 (-) sense RNA is formed by recombination in insect cells between

pAcUW31.TB2 and a baculovirus such as AcNPV to form AcNPV.TB2. However, the transfer vector may be any transfer vector containing baculovirus promotors, such as Pol and p10.

Production of the rabies virus VLPs containing a (-) sense RNA modified genome or genome fragment are produced by co-infection of an insect cell with the recombinant baculovirus AcNPV.TB2 and other recombinant baculoviruses which express rabies virus L protein, G protein, N protein, M1 protein and M2 protein as shown in Figure 4.

The procedure for preparation of recombinant baculoviruses which express rabies virus G and N proteins has been described in Prehaud *et al.* (1989) *Virology 173*, 390-399 and Prehaud *et al.* (1990) *Virology 178*, 486-497, and the baculovirus expression of rabies N, M1, M2 and G proteins is disclosed in Prehaud *et al.* (1992) *Virology 189*, 766-770, the entire disclosures of which are incorporated herein by cross-reference. Similar procedures can be used to prepare recombinant baculoviruses which express the rabies virus L protein in insect cells. The sequence of the L gene has been disclosed in Tordo *et al.* (1988) *Virology 165*, 565-576. From known gene sequences, a person of skill in the art can readily prepare vectors for the expression of proteins from other (-) sense RNA viruses.

As indicated above in step (i) of the overall process of the invention, one or more foreign genes are included in the DNA molecule. Using the TB-2 construct as an example, the construct can be modified to incorporate any selected foreign gene or genes by insertion of the selected gene or genes at the *Nco*I site. By using the *Nco*I site, the selected gene or genes can be positioned within the construct so that the initiation codon will substitute for the initiation codon of the nucleoprotein (N) gene of the virus from which the terminal domains are derived. However, the foreign gene or genes can be inserted at any suitable site within the filler domain, if present, or proximal the DNA sequences corresponding to the 5' or 3' domains of the (-) sense RNA genome.

Preferably, DNA comprising the foreign gene includes the initiation codon, termination codon and coding region of the selected foreign gene, all or a part of the 3' noncoding region

including the polyadenylation site of the rabies virus N protein mRNA, or equivalent sequence, and cohesive ends suitable for insertion of the DNA into the DNA molecule. As an example of the last mentioned feature, the DNA will have *NcoI* restriction termini for insertion of the DNA at the *NcoI* site of the TB-2 construct. It will be understood by one of skill in the art that the DNA comprising the foreign gene is inserted into the DNA molecule so that (+) sense RNA formed in a target cell contains the sense strand of the foreign gene.

The foreign gene can be obtained by established procedures of molecular cloning well known in the art. Addition of the 3' noncoding region, polyadenylation site and cohesive ends can be conducted, for example, by using PCR and suitable oligonucleotide primers which contain the desired sequences. Other methods of modification that are known in the art can also be used, such as ligation of oligonucleotide linkers to DNA comprising the gene.

It will be appreciated that the process described above for the production of rabies VLPs can be applied to any other (-) sense unsegmented RNA virus, particularly rhabdoviruses and paramyxoviruses. Essentially, the required VLP will contain a suitably modified genome or genome fragment containing a foreign gene or genes including essential assembly and transcription signals provided by the 5' and 3' domains of the DNA construct. Ribozyme domains R1 and R2 can be provided to ensure that the RNA transcript has suitable terminal sequences. The selected foreign gene can be inserted at any suitable site within the DNA construct. The RNA transcript of the DNA construct when co-expressed in eukaryotic cells with the structural proteins of the homologous (-) sense RNA virus is incorporated in a VLP.

As described in step (v) of the process of this invention, a VLP vector comprising a (-) sense genome which includes a foreign gene can be used to deliver the foreign gene to a eukaryotic cell and to express the polypeptide product or RNA of the foreign gene in the target cell.

It will also be appreciated that delivery and expression of the foreign gene in a eukaryotic cell will occur by adsorption of VLPs to specific receptors on the cell surface

and subsequent entry of the VLPs into the cytoplasm of the cell. Advantageously, expression of the foreign gene occurs by virtue of components of the ribonucleoprotein complex of the VLP which are activated in the cytoplasm of the target cell. In particular, the ribonucleoprotein complex contains an RNA-dependent RNA-polymerase such as the L protein of rabies virus. The presence of an RNA-dependent RNA-polymerase in the ribonucleoprotein complex is not essential however and the activity can be provided by co-transfection of the target cell with a vector from which an RNA-dependent RNA-polymerase activity is expressed. The vector may be a plasmid or a virus. Typically, the vector is an homologous (-) sense RNA virus.

It is known that recognition of and entry into cells by viruses is a function of the envelope glycoproteins on the viral surface. In the case of rabies virus and other (-) sense RNA viruses, this function is served by the G protein. The target cell specificity of the VLP vectors of the present invention can therefore be changed by modifying the structure of the envelope glycoprotein. This can be achieved by constructing chimeric envelope protein genes which can be substituted for the envelope glycoprotein gene(s) during step (iii) of the process described above. Methods for the construction and expression of chimeric viral glycoproteins are known and are described, for example, by Puddington *et al.* (*Proc. Natl. Acad. Sci USA 84*, 2756-2760 (1987)), Schubert *et al.* (*J. Virol. 66*, 1579-1589 (1992)) and Owens and Rose (*J. Virol. 67*, 360-365 (1993)).

A suitable method for the construction of a chimeric glycoprotein gene is illustrated in Figures 5a to 5d. In this illustration, the nucleotide sequence of a chimeric glycoprotein is constructed, for example, from the envelope glycoprotein genes of rabies virus and the rhabdovirus, vesicular stomatitis virus (VSV). The chimeric gene illustrated retains internal and transmembrane domains of the rabies glycoprotein but includes the external domain of VSV. The chimeric gene components are advantageously synthesised by PCR amplification of template DNA using oligonucleotide primers. Such primers are shown as "OLIGO 21" to "OLIGO 24" in Figures 5a and 5b. The chimeric glycoprotein gene can be substituted for the rabies G protein gene in an expression vector, a recombinant baculovirus for

example, and used for the construction of rabies VLP vectors. VLPs formed using such a chimeric structure will adsorb to and enter cells recognised by the VSV glycoprotein. Such a process can be used to construct chimeric envelope proteins which incorporate any selected external domain which can be included in the surface structure of the VLPs. The chimeric structure can be selected so that the VLPs can adsorb to, enter and express the foreign gene in specific cells which carry a receptor for the modified external domain.

External domains that can be used to alter the target cell specificity of the VLP vectors of this invention include, but are not limited to, the external domains of influenza virus hemagglutinin, human immunodeficiency virus (HIV) gp160, and paramyxovirus hemagglutinin-neuraminidase. Alternatively, the chimeric envelope protein can comprise an external domain from a virus fused to the trans-membrane and internal domains of the virus on which the VLP is based, wherein the first mentioned virus is different to the second mentioned virus.

In relation to step (ii) of the process described above, it will be appreciated by persons skilled in the art that any suitable vector-host cell system can be used to express the modified genome or genome fragment and viral proteins for VLP formation. Suitable host cells include higher eukaroytic cells such as vertebrate cells using poxvirus, papillomavirus or retrovirus vectors, or lower eukaryotic cells such as yeast cells. The preferred expression system is, however, an insect host cell such as *Spodoptera frugiperda* harbouring a recombinant baculovirus vector.

Following similar methods to those described above for expressing rabies VLPs based on the TB-2 construct, other (-) sense RNA genes can be expressed in insect cells using baculovirus vectors. Simultaneous expression of a genome construct as a (-) sense RNA transcript and homologous (-) sense RNA virus structural proteins in insect cells allows formation of VLPs.

For delivering a foreign gene to target cells of a subject, the VLP vectors of the invention may be administered as follows: by topical treatment of mucous membranes; by intramuscular, subcutaneous, intraperitoneal or intravenous

injection into tissue; or, by delivery to the intestinal mucosa either naked or in acid- and pepsin-resistant capsules. Examples of topical treatment of mucous membranes are oral, nasal, occular, respiratory, anal, vaginal or urethral routes. Alternatively, the VLP vectors may be administered to cells or tissue *in vitro* by directly contacting the cells or tissue with the VLPs.

Pharmaceutical preparations of the VLP vectors of the invention are prepared by combining the VLPs with pharmaceutically acceptable carriers, diluents, adjuvants or excipients or combinations thereof.

The number of VLPs administered to a target cell or target cell of a tissue will depend on the expression product of the foreign gene. In some instances a single VLP per cell will be sufficient whereas in other instances a large number of VLPs will be required per cell, such as, where the foreign gene expression product is an anti-sense RNA. One of skill in the art would be able to determine the number of VLPs to be administered from a consideration of the expression product of the foreign gene.

By using the process described in this invention, and illustrated using the rhabdovirus TB-2 genome, synthetic (-) sense RNA virus VLPs can be produced without helper virus, defective-interfering particles or synthetic transcription complexes. The VLPs synthesised by this process are modified to contain a foreign gene which can be delivered to and expressed in eukaryotic cells. The VLP vectors of the invention can be modified to include an external domain whih allows adsorption to and entry into cells of a selected type. The VLPs do not contain complete genes from the homologous (-) sense RNA virus so the synthetic particles are non-infectious.

The invention will now be illustrated by the following non-limiting examples. Except as otherwise noted, standard methods were used for the isolation and manipulation of nucleic acids described, for example, by Sambrook *et al.* in *Molecular Cloning: a Laboratory Manual* 2nd Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA (1989).

EXAMPLE 1

Preparation of recombinant plasmids containing VLP genome

constructs incorporating the chloroamphenicol acetyl transferase (CAT) reporter gene.

In this example, construction of plasmids comprising a sub-genomic fragment of rabies virus harbouring a foreign gene is described. The TB-2 construct described in international application No. PCT/AU92/00363 was utilised as a sub-genomic fragment of rabies virus and the CAT reporter gene employed as a foreign gene.

Plasmids used for constructing the recombinant plasmids were obtained from the following sources: pSVL-CAT (Cameron and Jennings (1989) *Proc. Natl. Acad. Sci. USA* 86, 9139) was obtained from CSIRO Division of Biomolecular Engineering, North Ryde, NSW, Australia; pBluescript KSII+ was obtained from Promega Corporation, Madison, WI, USA; and, pAcYM1 (Matsuura *et al.* (1987) *J. Gen. Virol.* 68, 1233) was obtained from the Institute of Virology and Environmental Microbiology, Oxford, UK.

Plasmid pTB2 is described in international application No. PCT/AU92/00363. The BamHI TB-2 insert of pTB2 is also contained within the pAcTB2 vector described in PCT/AU92/00363. pAcTB2 has been deposited with the Australian Government Analytical Laboratories, 1 Suakin Street, Pymble, NSW 2073, Australia, under accession No. 92/32588.

Oligonucleotide primers were synthesised for PCR amplification of required DNA fragments (CPR1, CPR2 and CPR3) and DNA sequencing (Bac1 and Bac2). CPR1, CPR2 and CPR3 contained the terminal sequences of the CAT gene, *NcoI* restriction endonuclease sites to facilitate subcloning into the *NcoI* site of pTB2 and in the case of CPR3, the rabies virus N-gene transcription termination/ polyadenylation sequence ($CATG[A]_7$). The sequence of each oligonucleotide primer follows:

CPR1 5'-CCCCATGGAGAAAAAAATCACTGGAT-3'

CPR2 5'-GGCCATGGTTACGCCCCGCCCTGC-3'

CPR3 5'-GGCCATGGTTTTTTTCATGTTACGCCCCGCCCTGC-3'

Bac1 5'-TTACTGTTTTCGTAACA-3'

Bac2 5'-CGCACAGAATCTAGCGC-3'.

A. Construction of plasmids pAcTB2-CAT and pAcTB2-CAT-R.

A full-length copy of the CAT gene was obtained by PCR using primers CPR1 and CPR2 and plasmid pSVL-CAT DNA as a

template for amplification. The reaction was performed using *Taq* Buffer, 3.5 mM $MgCl_2$, 0.25 mM of each dNTP, 5 units of *Taq* DNA polymerase (Promega Corp.), 1 µg of each primer and 7.5 ng of pSVL-CAT plasmid DNA. The reaction mixes were heated at 85°C, 3 min before the addition of *Taq* DNA polymerase and subjected to 40 cycles at 95°C for 90 s, 51°C for 90 s, 72°C for 90 s followed by incubation at 72°C for 5 min before maintainance at 25°C until DNA products were processed.

The CAT DNA product was applied to a 0.8% LMT agarose gel and a discrete DNA band of approximately 0.7 kb was excised. An equal volume of TE buffer pH 7.6 was added and the mixture was incubated at 68°C for 5-8 min with occasional vortexing. The DNA was extracted once with phenol, twice with phenol:chloroform:isoamyl alcohol (25:24:1) and was precipitated by the addition of 0.3 M sodium acetate pH 5.2, 20 mg glycogen (Boehringer Mannheim) as a carrier and 2.5 vol of ethanol. After incubation at -20°C for 30 min, DNA was collected by microcentrifugation, washed with 70% ethanol and dried under vacuum.

3'-Terminal adenosine overhangs resulting from *Taq* DNA polymerase extension were removed using the 3'->5' exonuclease activity associated with the Klenow fragment of DNA polymerase 1. Purified DNA products were reacted with 2.5 units of Klenow fragment (Promega Corp.) in restriction enzyme buffer H (Boehringer Mannheim) at 22°C for 15 min and extracted with phenol:chloroform:isoamyl alcohol and precipitated as above.

The CAT gene DNA was blunt-end ligated into the dephosphorylated *Eco*RV site of pBluescript KSII+ (Stratagene) followed by transformation of XL1-Blue *E. coli* host cells (Stratagene) and the selection of ampicillin-resistant white colonies on agar plates prepared with TYM medium and containing ampicillin, X-gal and IPTG. Plasmids containing the CAT gene were identified and the sequences of the inserts in pBlue-CAT were determined using T3 and T7 sequencing primers (Promega Corp.) and Sequenase™ (United States Biochemicals) sequencing reagents.

Plasmid pBlue-CAT was subjected to partial digestion with *Nco*I and the resulting DNA fragments were resolved in a 1.2% LMT agarose gel. The full-length CAT gene fragment of

approximately 0.7 kb was isolated, purified and subcloned into the dephosphorylated *NcoI* site of pTB2 as described above with the exception that no blue/white selection system was available. Plasmids containing inserts were identified and sequenced using the T7 sequencing primer to determine the orientation of inserts in the *NcoI* site of the TB2 genome construct. Two clones containing the CAT gene in forward (pTB2-CAT) and reverse (pTB2-CAT-R) orientation were selected as shown in Figure 6.

Plasmids pTB2-CAT and pTB2-CAT-R were digested with *BamHI* to obtain inserts containing the TB2 DNA construct incorporaiting the CAT gene in both orientations. The inserts of approximately 2.1 kb were isolated and purified as above and subcloned into the dephosphorylated *BamHI* site of the baculovirus transfer vector pAcYM1. Recombinant plasmids were identified and the orientation of the inserts was determined by sequencing as described above with primers Bac1 and Bac2 which allowed strand extension across the two reformed *BamHI* sites of the recombinant pAcYM1 vector. The sequences of the Bac1 and Bac2 primers are shown above. Clones possessing inserts in the required orientation, pAcTB2-CAT and pAcTB2-CAT-R, were selected and plasmid DNA purified by CsCl gradient centrifugation.

The construction of pAcTB2-CAT and pAcTB2-CAT-R is shown schematically in Figure 6.

B. Construction of plasmids pAcTB2-CAT3 and pAcTB2-CAT3-R.

Plasmids pAcTB2-CAT3 and pAcTB2-CAT3-R were prepared exactly as described above for plasmids pAcTB2-CAT and pAcTB2-CAT-R except that the CAT gene was obtained from plasmid pSVL-CAT DNA by PCR amplification using primers CPR1 and CPR3. This resulted in the inclusion of the rabies virus polyadenylation sequence ($CATG[A]_7$) immediately after the CAT gene termination codon in addition to the polyadenylation sequence present in TB-2.

The sequence of the CAT gene inserts in plasmids pTB2-CAT and pTB2-CAT3 are shown in Figure 7. Plasmid and primer sequences outside of the terminal *NcoI* restriction endonuclease sites are not presented.

EXAMPLE 2

Construction of recombinant baculoviruses containing TB2-CAT genome constructs.

The baculovirus AcPAK6 was grown in *Spodoptera frugiperda* (Sf9) cells and purified by sucrose gradient centrifugation. DNA was isolated and purified by CsCl gradient centrifigation and digested to completion with *Bsu*36I. Sf9 cells were co-transfected with 100 ng *Bsu*36I-linearized AcPAK6 DNA and 1 µg of each of the four plasmid constructs (pAcTB2-CAT, pAcTB2-CAT-R, pAcTB2-CAT3 and pAcTB2-CAT3-R) using Lipofectin™ (Gibco/BRL) transfection reagent. Cells were incubated at 28°C for 4 days and recombinant baculoviruses were identified by plaque selection. Cells were treated with X-gal to differentiate wild type (blue) from recombinant (white) baculovirus clones and the plaques were visualized by staining with neutral red. Clearly defined white plaques were selected and grown in duplicate 96-well cultures of Sf9 cells at 28°C for 3-6 days.

Cell lysates were prepared from one set of duplicate cultures after 3 days for hybridization analyses to confirm integration of the four TB2-CAT genome constructs. A digoxigenin (DIG)-labelled probe was prepared by PCR using 12 ng gel-purified CAT3 PCR product as a template, 0.5 µg CPR1 and CPR3 primers, reaction mixes containing 4mM $MgCl_2$, 0.5 mM dATP, dCTP and dGTP, 0.32 mM dTTP, 8 nmol DIG-11-dUTP (Boehringer Mannheim), 2.5 units *Taq* DNA polymerase (Promega Corp.) and the cycling temperatures described above. Dot hybridizations with the DIG-labelled CAT probe identified recombinant baculoviruses containing the TB2-CAT, TB2-CAT-R, TB2-CAT3 and TB2-CAT3-R genome constructs.

After 4 days, a portion of the medium from duplicate cultures infected with recombinant baculoviruses identified as containing the required genome constructs was used to infect 24-well cultures of Sf9 cells to produce virus seed stocks for subsequent infection of larger cell cultures. Cultures of Sf9 cells were infected as a source of DNA for PCR amplification to confirm that complete rather than truncated DNA constructs had integrated into the recombinant baculoviruses. DNA obtained from cell lysates was used as a template for PCR amplification with the Bac1 and Bac2 primers described above. Amplification products were resolved in 0.8% agarose gels and fragments of the appropriate size, approximately 2.1 kb, were identified

confirming that the recombinant baculoviruses contained full length TB2-CAT, TB2-CAT-R, TB2-CAT3 and TB2-CAT3-R constructs.

To produce cloned virus stocks, virus seed stocks from the 24-well cultures were subjected to a second round of plaque purification in Sf9 cells. Well- separated plaques were selected, isolated and used to produce stocks of cloned recombinant baculoviruses for use in subsequent manipulations.

EXAMPLE 3

Preparation of rabies VLPs containing TB2-CAT genome constructs.

Recombinant baculoviruses expressing rabies virus structural proteins (N/M1 and M2/G in dual expression vectors) and each of the four recombinant baculoviruses expressing genome constructs TB2-CAT, TB2-CAT-R, TB2-CAT3 and TB2-CAT3-R were used to infect spinner cultures of Sf9 cells. Cultures were incubated at 28°C for 3 days, the medium harvested, clarified by centrifugation and VLPs were collected by ultracentrifugation at 27000 rpm for 1 h at 4°C in a Beckman SW28 rotor. VLPs were resuspended in TD buffer supplemented with 1 mM EDTA and centrifuged through TD-buffered 10% (w/w) sucrose onto a cushion of TD-buffered 40% (w/w) sucrose at 35000 rpm for 30 min at 4°C in a Beckman SW40Ti rotor. The band at the interface was harvested, diluted and the semi-purified VLPs collected by centrifugation at 30000 rpm for 90 min at 4°C in a Beckman SW40Ti rotor.

VLP formation was demonstrated by SDS-PAGE of disrupted pellets and Western blotting using polyclonal rabies virus antiserum. Rabies virus structural proteins G, N, M1 and M2 were identified in VLPs produced with all four TB2 genome constructs containing the CAT gene - TB2-CAT, TB2-CAT-R, TB2-CAT3 and TB2-CAT3-R. Visual comparison of the intensity of the structural proteins in VLPs produced using the four TB2-CAT genome constructs with that observed for VLPs produced with the TB2 genome suggested that the Sf9 cells shed similar quantities of VLPs irrespective of the nature of the TB2 genome employed.

EXAMPLE 4

Detection of CAT gene expression.

In this example, expression of the CAT reporter gene in target cells transfected with the VLPs prepared in Example 3

is described. As the VLPs did not contain the L gene product, cells were co-transfected with live rabies virus to provide RNA-dependent RNA-polymerase activity. The (-) sense RNA of the VLPs was thus converted to (+) sense RNA allowing CAT expression.

Experiment 1.

Monolayers of 5 x 10^5 baby hamster kidney cells (BHK-21, BSR clone) were infected with 8 x 10^6 plaque-forming units of rabies virus (CVS strain). At 4 hours post-infection, the infected monolayers and uninfected BHK-21 cell monolayers were treated with 2 x 10^9 VLPs containing the following genome constructs: (a) TB2-CAT; (b) TB2-CAT3; (c) TB2-CAT-R; (d) TB2-CAT3-R; or, (e) no VLPs. At 2 days post-infection all monolayers were harvested and assayed for CAT gene expression by using the CAT-ELISA (Boehringer Mannheim).

Experiment 2.

In a second experiment, BHK-21 cell monolayers were treated as described in Experiment 1 except that rabies virus infections and VLP treatments were conducted simultaneously: that is, a 4 hour interval was not allowed between infection and VLP treatment.

The results of both experiments are presented in Table I.

TABLE I.

CAT gene expression from VLPs in BHK-21 cells (BSR clone) as detected by CAT-ELISA.

VLP Genome	Quantity of CAT expressed (pg)			
	Experiment 1		Experiment 2	
	Rabies-infected cells	Uninfected cells	Rabies-infected cells	Uninfected cells
TB2-CAT	15	0	15	0
TB2-CAT3	15	0	15	0
TB2-CAT-R	0	0	0	0
TB2-CAT3-R	0	0	0	0
No VLPs	0	0	0	0

The results presented in Table I demonstrate that a foreign gene, in this case CAT, can be expressed from sequence information contained within a (-) sense VLP genome. Expression was dependent on correct orientation of the foreign gene with CAT being detectable only in those cells transfected with TB2-CAT and TB2-CAT3 VLP genomes. Expression was also dependent on viral RNA-dependent RNA-polymerase activity as CAT was only detectable in cells infected with rabies virus (left-hand column for each experiment in Table I).

It will be appreciated that administration of the VLPs of the invention which can express an immunogenic protein to animals or humans who are at risk of disease, infection or infestation will cause immunity in much the same way as existing vaccines incorporating inactivated / or attenuated viruses. However, there will be advantages because there is no possibility that infectious virus will be present or that reversion to virulence will occur because the VLPs of the present invention use only a fragment of the viral genome. Similarly, the VLPs of this invention can be used to deliver therapeutic agents to diseased or infected tissue. However, unlike other delivery systems presently available, the VLPs can be targeted to specific cells or tissues, can allow synthesis and hence amplification of the therapeutic agent in the target tissue, are completely non-infectious and typically do not carry genes of an infectious agent. Moreover, as the VLPs contain no DNA, no integration elements and no enzyme capable of DNA synthesis, there is no risk of modification of the host genome which can result in the induction of tumours or related disorders.

It will also be appreciated that many modifications can be made to the invention described above without departing from the broad scope and ambit thereof.

DEPOSITION OF MATERIAL ASSOCIATED WITH THE INVENTION

A sample of plasmid pAcTB2 was deposited with the Australian Government Analytical Laboratories, 1 Suakin Street, Pymble, NSW 2073, Australia, on 15 September 1992 and given the accession number 92/32588.

CLAIMS

1. A vector for delivering a foreign gene to a target cell for expression of said foreign gene, said vector comprising a (-) sense RNA genome contained within a ribonucleoprotein complex within a virus-like particle constituted from structural proteins of a (-) sense RNA virus, wherein said (-) sense RNA genome includes one or more foreign genes but does not include genes for replication of said (-) sense RNA virus.

2. A vector according to claim 1 which further comprises within said ribonucleoprotein complex a polymerase for synthesis of (+) sense RNA from said (-) sense RNA genome.

3. The vector according to claim 2 wherein said (-) sense RNA virus is a rhabdovirus or paramyxovirus.

4. The vector according to claim 3 wherein said (-) sense RNA virus is rabies virus.

5. The vector according to claim 4 wherein:

said (-) sense RNA genome comprises a 5' domain from the genome of rabies virus, a filler domain comprising rabies virus genomic RNA, said one or more foreign genes and a 3' domain from the genome of rabies virus;

said ribonucleoprotein complex comprises said (-) sense RNA genome together with rabies M1 and L proteins surrounded by a sheath of rabies N protein; and

said ribonucleoprotein complex is surrounded by an internal matrix comprising rabies M2 protein and is enclosed in a lipid envelope including rabies G protein.

6. A vector according to claim 1 wherein said virus-like particle includes modified glycoprotein comprising an external domain which targets said virus-like particle to a selected cell type.

7. The vector according to claim 6 wherein said modified glycoprotein comprises the internal and transmembrane domains of rabies virus G protein fused to an external domain comprising a polypeptide ligand for a receptor on the surface of said selected cell type.

8. The vector according to claim 1 wherein the expression product of said foreign gene is selected from the group consisting of a peptide, a polypeptide, an anti-sense RNA and a catalytic RNA.

9. A method of preparing a vector for delivering a foreign gene to a target cell for expression of said foreign gene, said vector comprising a (-) sense RNA genome contained within a ribonucleoprotein complex within a virus-like particle constituted from structural proteins of a (-) sense virus, wherein said (-) sense RNA genome includes one or more foreign genes but does not include genes for replication of said (-) sense RNA virus, which method comprises the following steps:

i) preparing an expression vector incorporating a DNA molecule which contains DNA corresponding to said (-) sense RNA genome;

ii) introducing the expression vector prepared in step (i) into a eukaryotic host cell together with DNA for the expression of proteins for the formation of virus-like particles;

(iii) culturing the eukaryotic host cells under conditions which allow expression of said (-) sense RNA genome and said proteins, and incorporation of said (-) sense RNA genome into virus-like particles; and

(iv) harvesting said virus-like particles from the eukaryotic cell culture of step (iii).

10. The method according to claim 9 wherein said DNA molecule includes at least one ribozyme domain which cleaves the initial (-) sense RNA transcript formed in step (iii) to provide a molecule which can be incorporated into said virus-like particles.

11. A method according to claim 10 wherein said DNA molecule includes two ribozyme domains which cleave the initial (-) sense RNA transcript to, provide a molecule having 5' and 3' ends which approximate the 5' and 3' ends of the genome of said (-) sense RNA virus.

12. The method according to claim 9 wherein said expression vector is derived from a baculovirus.

13. The method according to claim 12 wherein said baculovirus is AcNPV or AcPAK6.

14. The method according to claim 9 wherein said eukaryotic host cell is an insect cell.

15. The method according to claim 14 wherein said insect cell is *Spodoptera frugiperda*.

16. A pharmaceutical composition comprising a

pharmaceutically acceptable carrier, diluent, adjuvant and/or excipient together with a vector for delivering a foreign gene to a target cell for expression of said foreign gene, said vector comprising a (-) sense RNA genome contained within a ribonucleoprotein complex within a virus-like particle constituted from structural proteins of a (-) sense virus, wherein said (-) sense RNA genome includes one or more foreign genes but does not include genes for replication of said (-) sense RNA virus, and wherein said ribonucleoprotein complex includes a polymerase for synthesis of (+) sense RNA from said (-) sense RNA.

17. A method of delivering the expression product of a foreign gene to a target cell, said method comprising contacting said target cell with a vector according to claim 1 and co-transforming or co-transfecting said cell with a vector which provides a RNA-dependent RNA polymerase activity.

18. A method of delivering the expression product of a foreign gene to a target cell, said method comprising contacting said target cell with a vector according to claim 2.

19. A method of delivering the expression product of a foreign gene to cells of a tissue of a mammalian subject, said method comprising administering to said subject a vector according to claim 2 or a pharmaceutical composition according to claim 16.

20. The method according to claim 19 wherein said delivery of the expression product of a foreign gene is for the treatment of a disease state or a pathological condition.

21. The method according to claim 19 wherein said virus-like particle includes modified glycoprotein comprising an external domain which targets said virus-like particle to a selected cell type.

22. The method according to claim 21 wherein said modified glycoprotein comprises the internal and transmembrane domains of rabies virus G protein fused to an external domain comprising a polypeptide ligand for a receptor on the surface of said selected cell type.

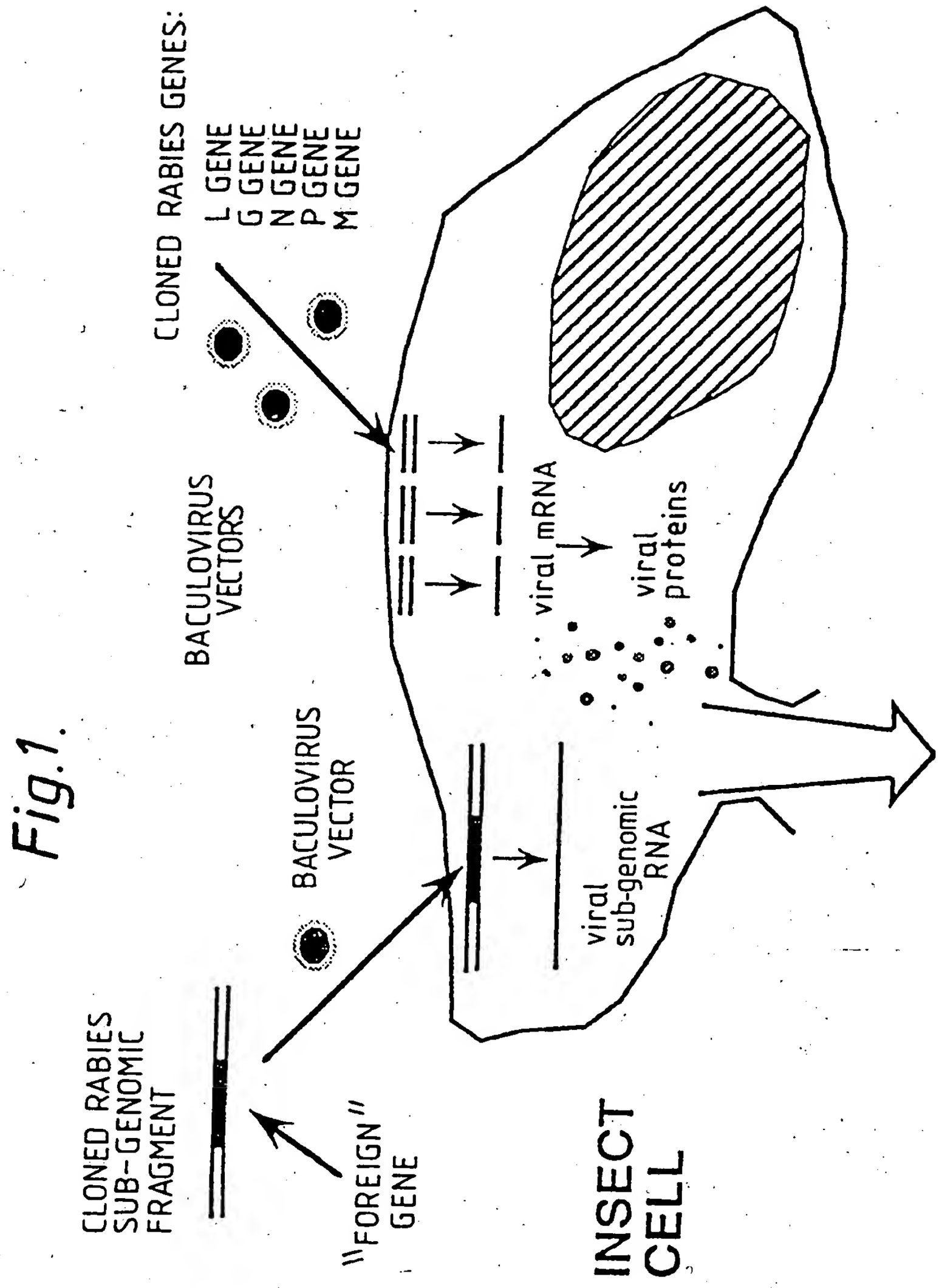


Fig.1.

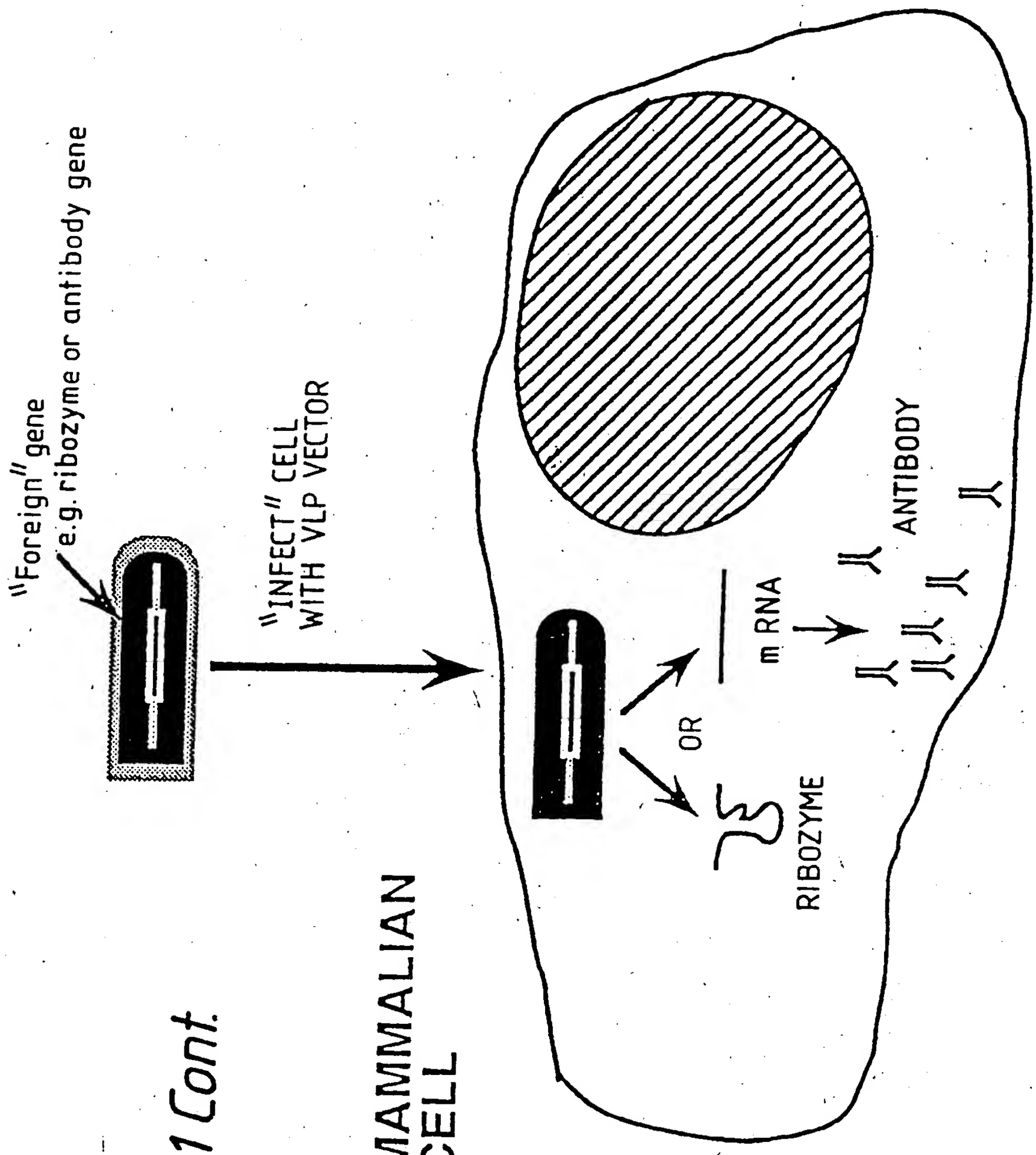


Fig.1 Cont.

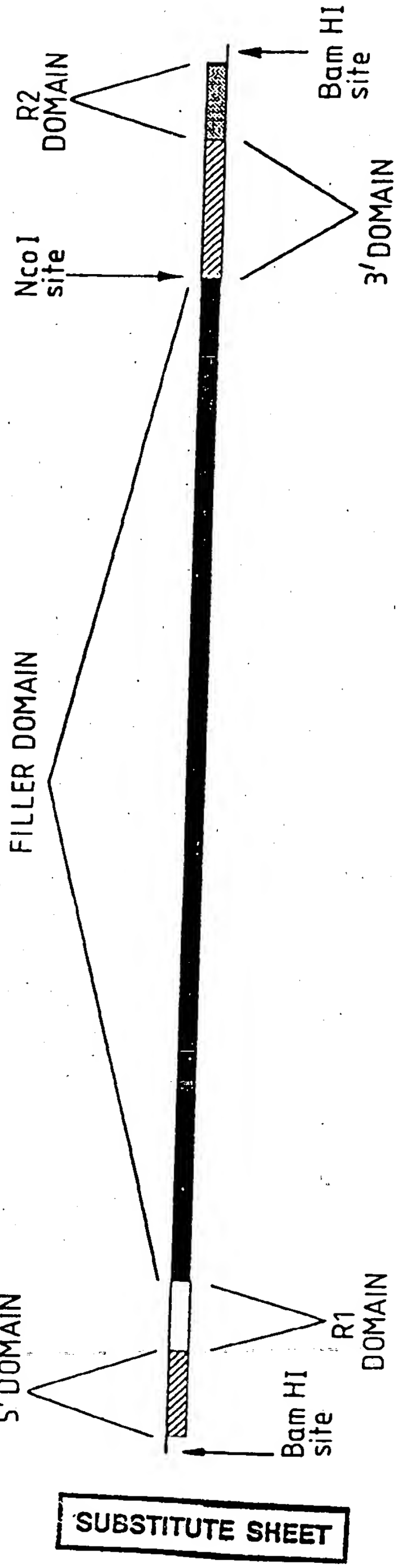


Fig.2.

Fig. 3.

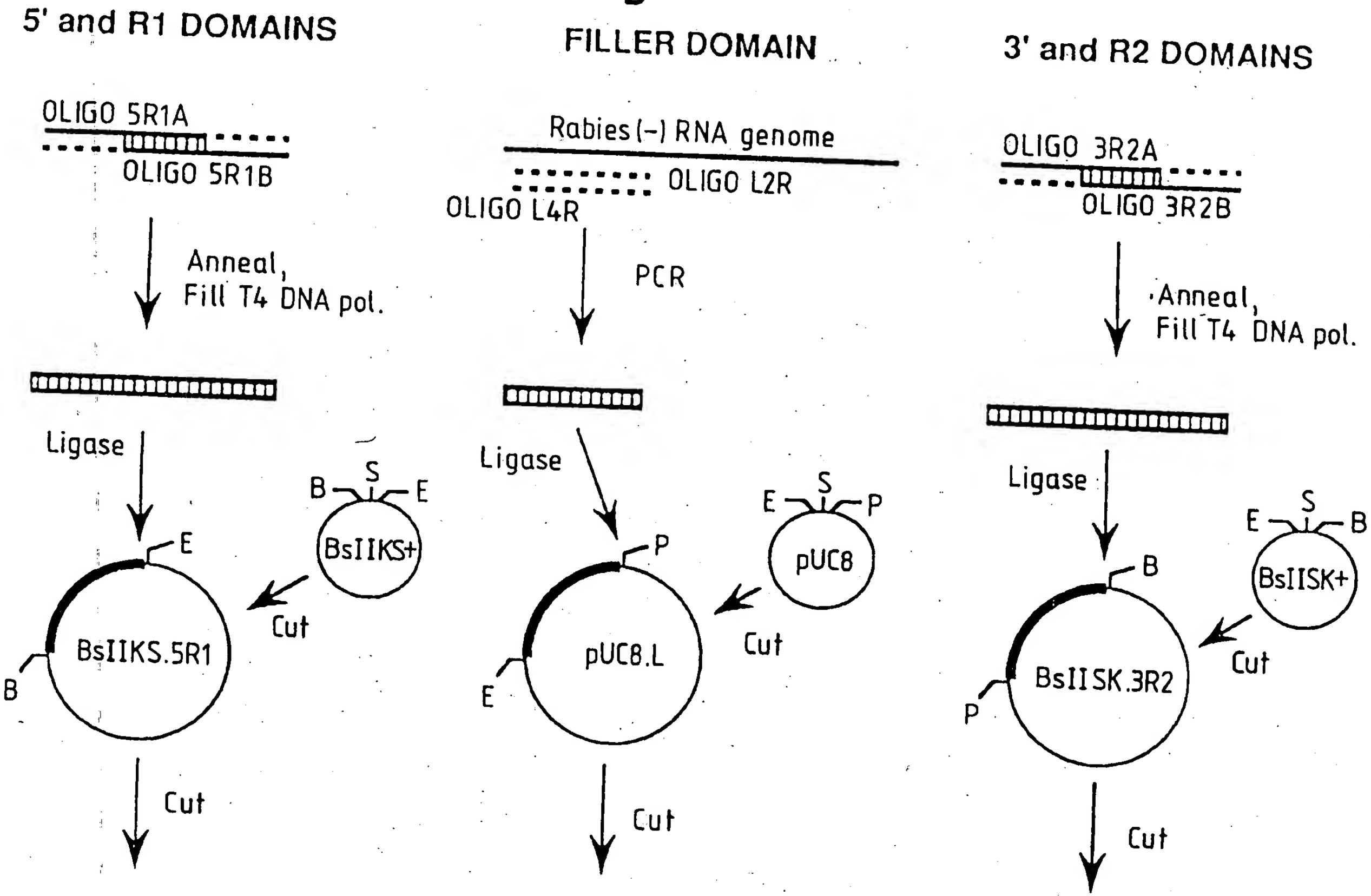


Fig.3Cont.

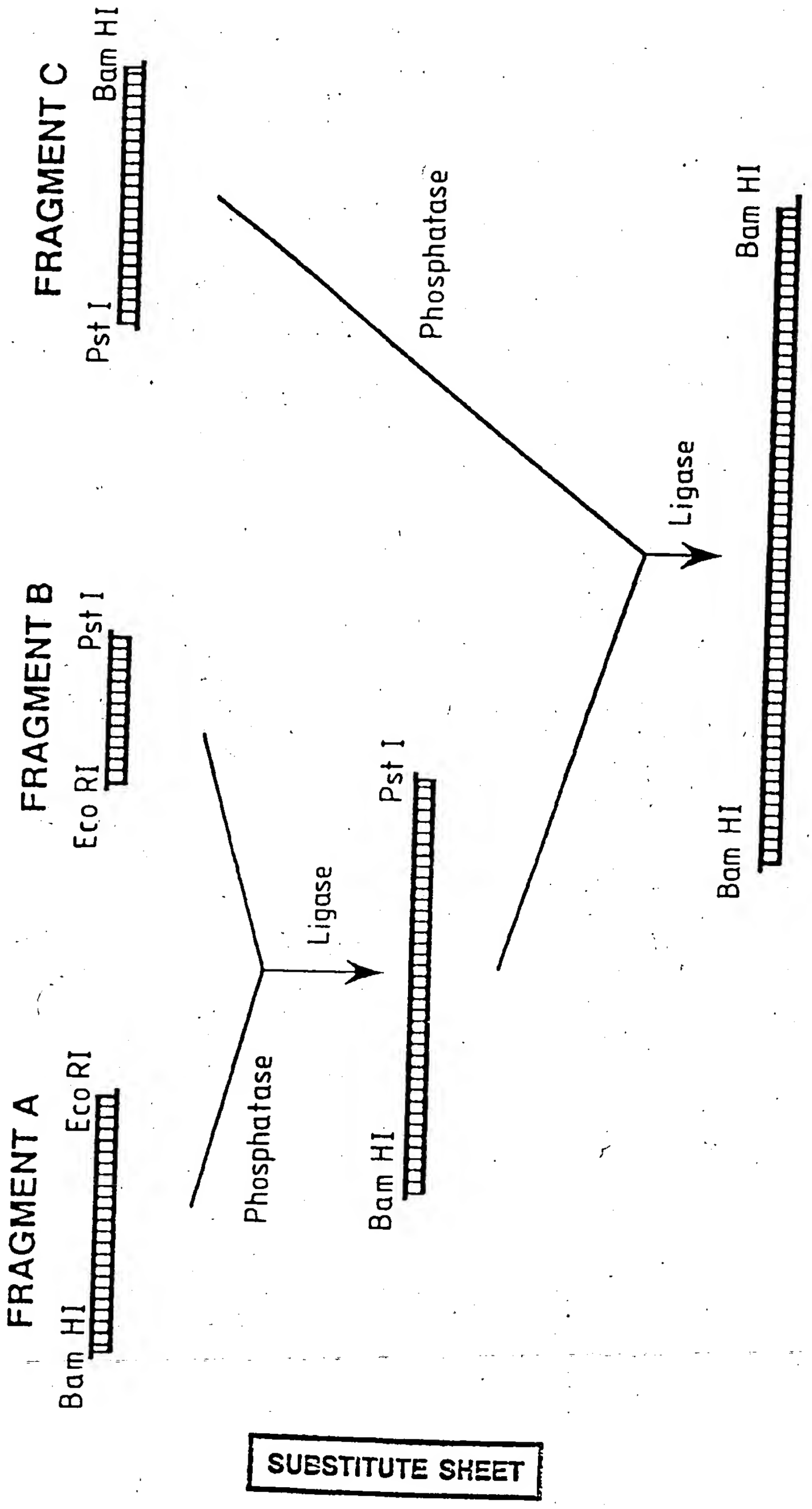


Fig.4.

Fig.5a.

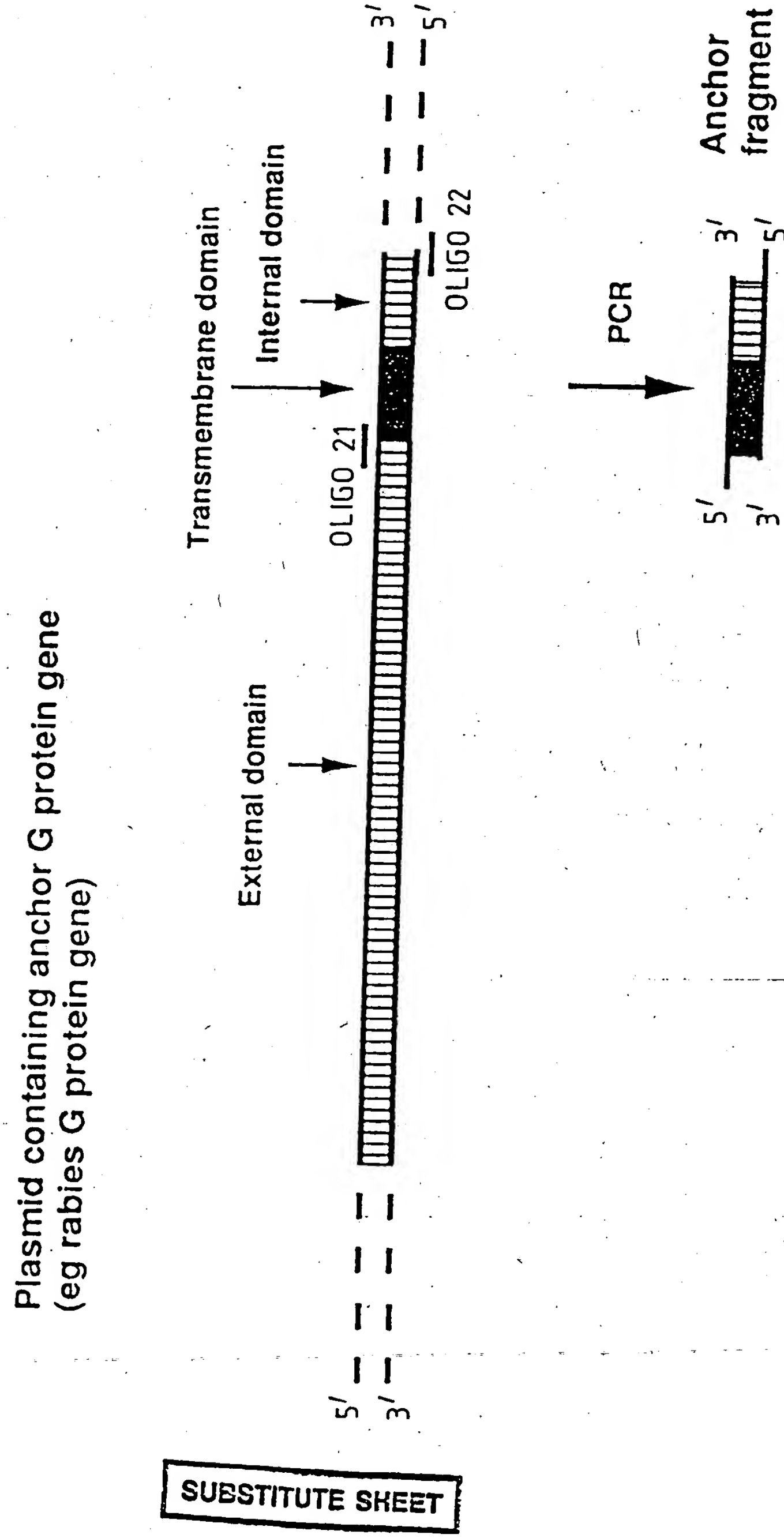


Fig.5b.

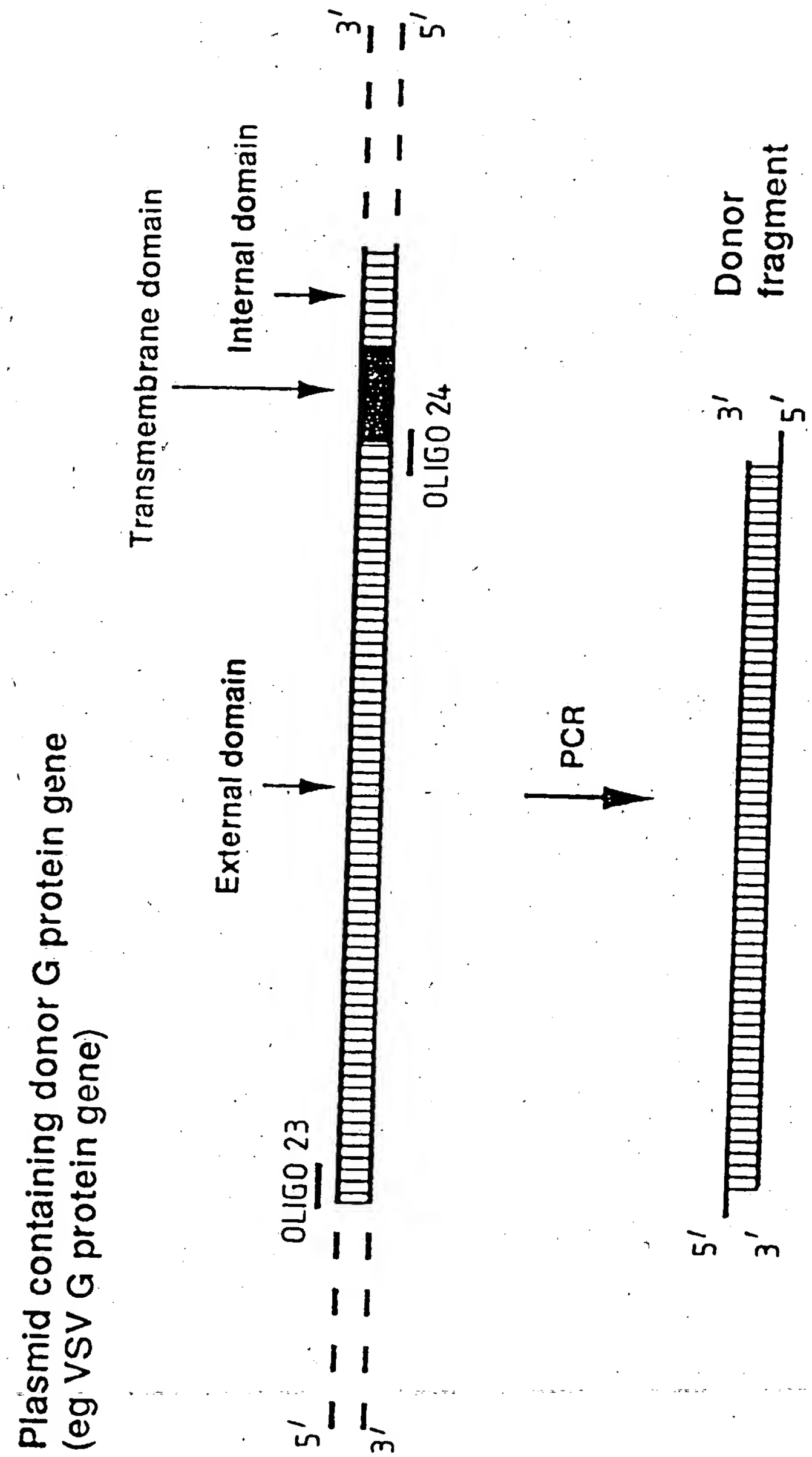
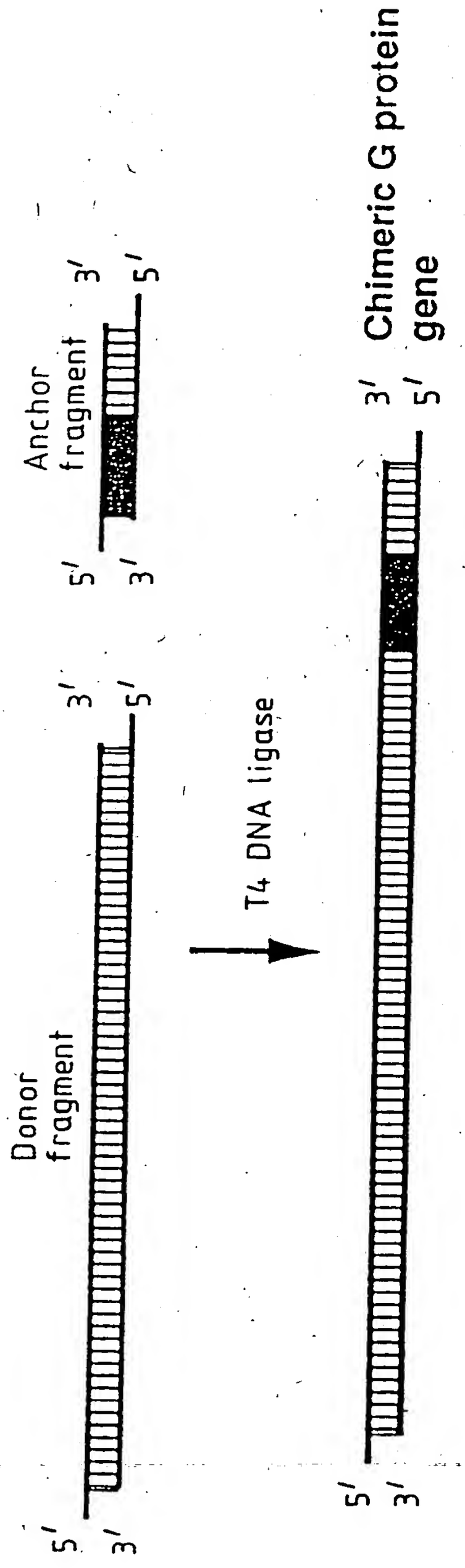


Fig.5c.


Anchor fragment
5′
3′
3′
5′
T4 DNA ligase
Donor fragment
3′
5′
5′
3′
3′ Chimeric G protein gene
5′
5′
3′

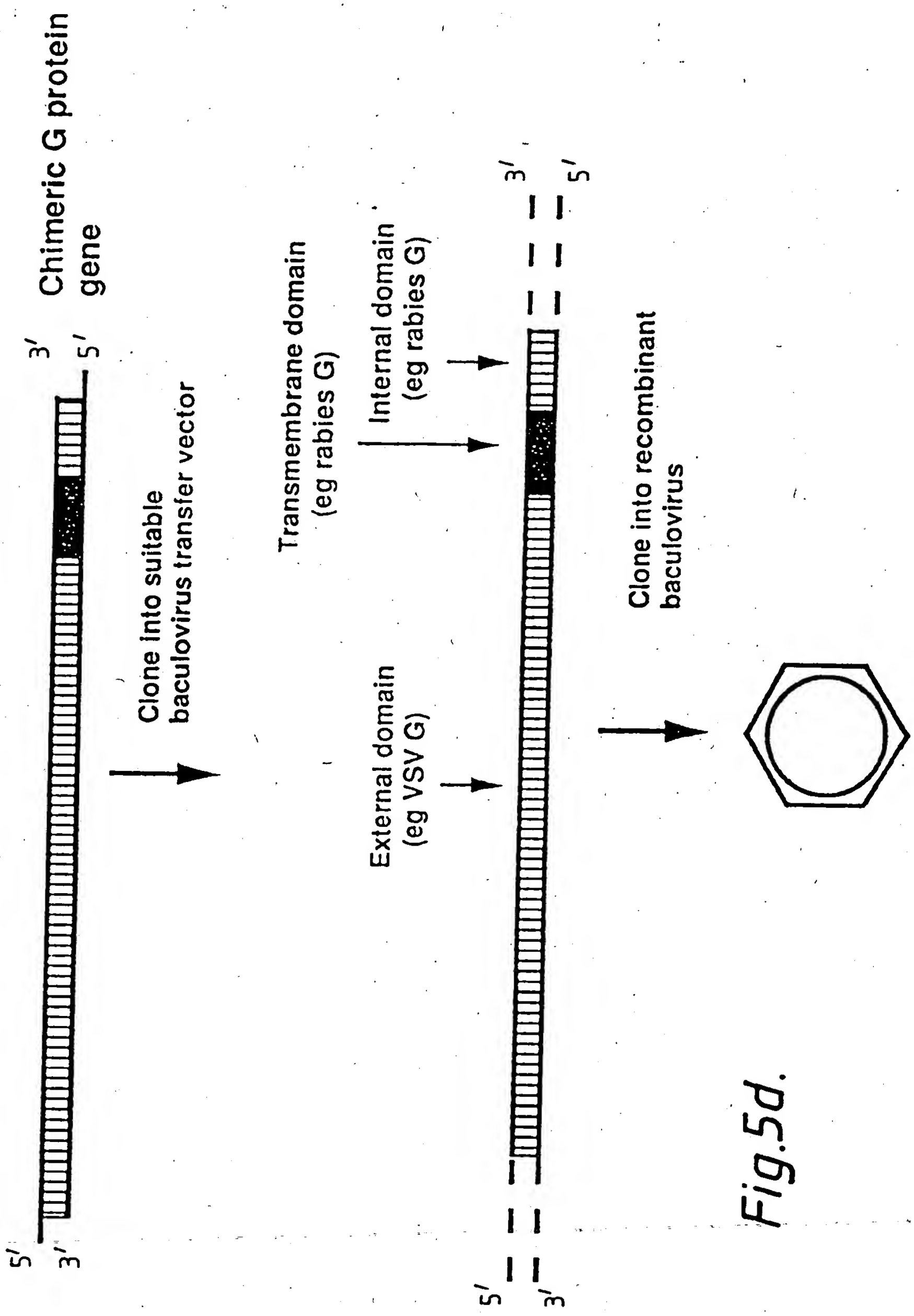
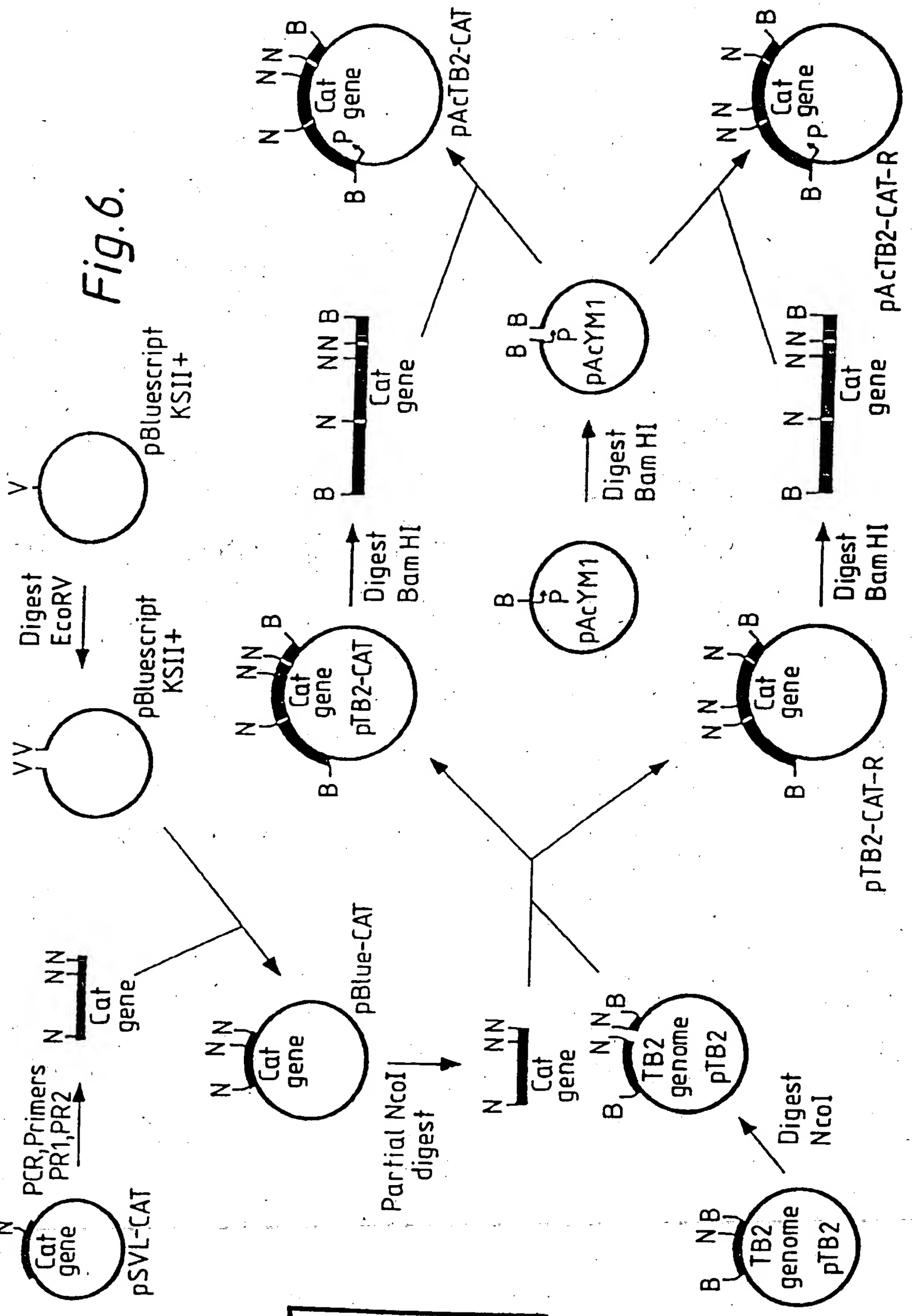

Chimeric G protein gene
3'
5'
Clone into suitable baculovirus transfer vector
Transmembrane domain (eg rabies G)
Internal domain (eg rabies G)
External domain (eg VSV G)
Clone into recombinant baculovirus
5'
3'

Fig.5d.

Fig. 6.



```
     NcoI
5'-CC ATG GAG AAA AAA ATC ACT GGA TAT ACC ACC GTT GAT ATA TCC CAA   47
      Met Glu Lys Lys Ile Thr Gly Tyr Thr Thr Val Asp Ile Ser Gln
                      5                  10                  15

TGG CAT CGT AAA GAA CAT TTT GAG GCA TTT CAG TCA GTT GCT CAA TGT   95
Trp His Arg Lys Glu His Phe Glu Ala Phe Gln Ser Val Ala Gln Cys
                  20                  25                  30

ACC TAT AAC CAG ACC GTT CAG CTG GAT ATT ACG GCC TTT TTA AAG ACC  143
Thr Tyr Asn Gln Thr Val Gln Leu Asp Ile Thr Ala Phe Leu Lys Thr
              35                  40                  45

GTA AAG AAA AAT AAG CAC AAG TTT TAT CCG GCC TTT ATT CAC ATT CTT  191
Val Lys Lys Asn Lys His Lys Phe Tyr Pro Ala Phe Ile His Ile Leu
          50                  65                  70

GCC CGC CTG ATG AAT GCT CAT CCG GAA TTC CGT ATG GCA ATG AAA GAC  239
Ala Arg Leu Met Asn Ala His Pro Glu Phe Arg Met Ala Met Lys Asp
      75                  80                  85

GGT GAG CTG GTG ATA TGG GAT AGT GTT CAC CCT TGT TAC ACC GTT TTC  287
Gly Glu Leu Val Ile Trp Asp Ser Val His Pro Cys Tyr Thr Val Phe
  90                  95                  100                 105
```

Fig.7a.

```
CAT GAG CAA ACT GAA ACG TTT TCA TCG CTC TGG AGT GAA TAC CAC GAC 335
His Glu Gln Thr Glu Thr Phe Ser Ser Leu Trp Ser Glu Tyr His Asp
                110                 115                 120

                                    T
GAT TTC CGG CAG TTT CTA CAC ATA TAT TCG CAA GAT GTG GCG TGT TAC 383
Asp Phe Arg Gln Phe Leu His Ile Tyr Ser Gln Asp Val Ala Cys Tyr
            125                 Leu 130                 135

GGT GAA AAC CTG GCC TAT TTC CCT AAA GGG TTT ATT GAG AAT ATG TTT 431
Gly Glu Asn Leu Ala Tyr Phe Pro Lys Gly Phe Ile Glu Asn Met Phe
        140                 145                 150

TTC GTC TCA GCC AAT CCC TGG GTG AGT TTC ACC AGT TTT GAT TTA AAC 479
Phe Val Ser Ala Asn Pro Trp Val Ser Phe Thr Ser Phe Asp Leu Asn
    155                 160                 165

                                                NcoI
GTG GCC AAT ATG GAC AAC TTC TTC GCC CCC GTT TTC ACC ATG GGC AAA 527
Val Ala Asn Met Asp Asn Phe Phe Ala Pro Val Phe Thr Met Gly Lys
170                 175                 180                 185

TAT TAT ACG CAA GGC GAC AAG GTG CTG ATG CCG CTG GCG ATT CAG GTT 575
Tyr Tyr Thr Gln Gly Asp Lys Val Leu Met Pro Leu Ala Ile Gln Val
                190                 195                 200
```

Fig.7b.

```
CAT CAT GCC GTT TGT GAT GGC TTC CAT GTC GGC AGA ATG CTT AAT GAA 623
His His Ala Val Cys Asp Gly Phe His Val Gly Arg Met Leu Asn Glu
          205                 210                 215

                                                    NcoI
                                                    CCATGG-3'
TTA CAA CAG TAC TGC GAT GAG TGG CAG GGC GGG GCG TAA CATGAAAAAA  672
Leu Gln Gln Tyr Cys Asp Glu Trp Gln Gly Gly Ala ***
     220                 225                 229

  NcoI
ACCATGG-3' 679
```

Fig.7c.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 93/00495

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl.[5] C12N 15/64, 15/86, A61K 48/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
ELECTRONIC DATABASES AS BELOW

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
IPC[5]: C12N 15/64, 15/86

Electronic data base consulted during the international search (name of data base, and where practicable, search terms used)
DERWENT DATABASES: WPAT, CHEMICAL ABSTRACTS, BIOTECHNOLOGY
KEYWORDS: (SENSE OR ANTISENSE) () RNA(S)(GENOM: OR VIRUS: OR VIRAL OR GENE#) PLUS (AND VECTOR# OR EXPRESS:) IN CHEMICAL ABSTRACTS AND BIOTECHNOLOGY DATABASES

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X Y	K YAMANKA et al: "In vivo analysis of the promoter structure of the influenza virus RNA genome using a transfection system with an engineered RNA". Proc Natl Acad Sci USA, volume 88, pages 5369-5373, June 1991. See entire document.	1, 2, 8, 9-11, 17, 18 8, 9
Y	W LUYTJES et al: "Amplification, Expression, and Packaging of a Foreign Gene by Influenza Virus" Cell, volume 59, pages 1107-1113, 22 December, 1989. See abstract	1, 8, 9

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 December 1993 (23.12.93)	13 JAN 1994 (13.01.94)
Name and mailing address of the ISA/AU AUSTRALIAN INDUSTRIAL PROPERTY ORGANISATION PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No. 06 2853929	Authorized officer for **ROBYN PORTER** Telephone No. (06) 2832318

Form PCT/ISA/210 (continuation of first sheet (2)) (July 1992) copbko

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 93/00495

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate of the relevant passages	Relevant to Claim No.
Y	D E SLEAT et al: "Selective Recovery of foreign gene transcripts as virus-like particles in TMV-infected transgenic tobaccos". Nucleic Acids Research, volume 16, number 8, pages 3127-3140, (1988)	1
A	M J DICKINSON & A PRYOR: "Isometric virus-like particles encapsidate the double-stranded RNA found in Puccinia striiformis, Puccinia recondita, and Puccinia sorghi" Can J Bot, Volume 67, pages 3420-3425, (1989)	
A	M J DICKINSON & A J PRYOR: "Encapsidated and unencapsidated double-stranded RNAs in flax rust, Melampsora lini" Can J Bot, volume 67, pages 1137-1142, 1989	
A	H REVETS et al: "Identification of virus-like particles in Eimeria stiedae". Molecular & Biochemical Parasitology, volume 36, pages 209-216, 1989.	
P,A	AU,A, 23665/92 (COMMONWEALTH SCIENTIFIC AND INDUSTRIAL RESEARCH ORGANISATION) 23 February 1993 (23.02.93)	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU 93/00495

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically

claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Member
AU 23665	WO 9301833

END OF ANNEX

Form PCT/ISA/210(patent family annex)(July 1992)